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Host transcriptomic response to Newcastle disease virus in relatively resistant and susceptible inbred chicken lines

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

This dissertation is dedicated to my family; the one I was born into and the one I chose. Without their love and support, I would not be where I am today.

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ABSTRACT

Newcastle disease virus (NDV) has devastating impacts on poultry throughout the world. The inbred Fayoumi and Leghorn chicken lines, previously characterized as relatively resistant and susceptible, respectively, to various pathogens were utilized to characterize host response to the NDV vaccine and identify potential genes and pathways associated with NDV resistance. Three-week-old chicks were inoculated with La Sota NDV (challenged) or PBS (nonchallenged) via the oculonasal route. At 2, 6, and 10 days post infection (dpi), approximately one-third of the birds within each line and challenge group were euthanized for tissue collection. At 2 and 6 dpi, lachrymal fluid was collected from all birds for viral quantification using qPCR. At 0 and 10 dpi, serum was collected from all birds to quantify NDV antibody levels using ELISA. The transcriptome response in tracheal epithelial cells, lungs, and Harderian glands from four birds per treatment group (line, dpi, and challenge group; 48 birds total) was analyzed using RNA-seq. These three tissues chosen for their location near the site of infection. A separate study analyzed mean survival time after challenge with velogenic NDV. These studies found the Fayoumi and Leghorn chickens were relatively resistant and susceptible, respectively to lentogenic and velogenic NDV, based on lachrymal fluid viral load, antibody levels, and within-tissue viral transcript counts detected by RNA-seq after the lentogenic challenge, and survival analysis after a velogenic challenge.

The trachea, lung, and Harderian gland RNA-seq data were analyzed individually and jointly to gain a more comprehensive understanding of the impact of lentogenic NDV challenge using differential expression and co-expression analyses. The tissues with detectable viral transcripts, trachea and Harderian gland, activated T cell related pathways

after challenge. The challenge had the largest impact on the trachea transcriptome, based on principal component analysis and numbers of differentially expressed genes. In the lung and Harderian gland, especially, the two lines had distinct responses to the virus. The combined tissue analysis revealed clusters of co-expressed genes that were correlated with important factors like line, challenge status, and dpi. Overall, EIF2 signaling, mTOR signaling, collagen related genes, and TNFSF13B / TNFRSF13B were identified as potential candidate genes and pathways related to NDV resistance. Further studies are required to determine and confirm how these genes and pathways impact NDV resistance.

CHAPTER 1. INTRODUCTION

Organization

This dissertation is composed of six chapters and an appendix. The current chapter (Chapter 1. Introduction) includes a comprehensive literature review encompassing chickens, immunology, Newcastle disease virus, RNA-sequencing, and more. Chapter 2, entitled 'Novel mechanisms revealed in the trachea transcriptome of resistant and susceptible chicken lines following infection with Newcastle disease virus', has been published in Clinical and Vaccine Immunology. Chapter 3, entitled 'Resistant and susceptible chicken lines show distinctive responses to Newcastle disease virus infection in the lung transcriptome', has been published in BMC Genomics. Chapter 4, entitled 'Novel analysis of the Harderian gland transcriptome response to Newcastle disease virus in two inbred chicken lines', is currently under review for publication in Scientific Reports. Chapter 5, entitled 'Tissue transcriptome joint analysis after vaccine challenge in inbred chicken lines of differential resistance to virulent Newcastle disease virus', has been submitted for publication in PLoS ONE. The discussion in Chapter 6 includes a summary of all research chapters, synthesis of the results, and proposed future directions. Lastly, Appendix 1 includes a manuscript submitted for publication in Frontiers in Physiology (avian physiology special edition) entitled 'What makes the Harderian gland transcriptome different from other chicken immune tissues? A gene expression comparative analysis'. In total, 5 first author manuscripts are included in this dissertation. Melissa Deist also co-authored two additional manuscripts during her PhD work, which have been cited in the discussion.

Chickens - a food source and tool for scientific discovery

Chickens were domesticated in Southeast Asia from the jungle fowl, and studies predict multiple domestication events occurred (Liu et al. 2006; Eriksson et al. 2008). The dispersal pattern of chickens out of Asia over 3,000 years ago can provide useful information on the migration of humans (Storey et al. 2012). Chickens have played an important role throughout history in religion, art, entertainment, and food.

Chickens have been integral to many important discoveries in science, especially in immunology and development. The egg-packaged embryo enables researchers to study aspects of vertebrate development more easily than in mammals. The discovery and understanding of B cells was determined in the chicken (Cooper et al. 1966). Another study, showed that a viral gene originated from the host species (chicken) (Stehelin et al. 1976). The first licensed cancer vaccine was also developed in chickens (Davison and Nair 2005). Also, chickens were the first livestock species to have their genome sequenced. The chicken genome is three times smaller than the human genome (Consortium 2004). Chickens have 38 autosomes (2n=78) and a pair of sex chromosomes (ZW female, ZZ male).

Genes related to the immune system are historically under high selection pressure because they are critical for survival; therefore, although humans and chickens share many characteristics, after 310 million years of separation (Hedges 2002) their immune systems have unique properties. Unlike humans, chickens lack a lymphatic system (Dransfield 1944), have heterophils instead of neutrophils (Genovese et al. 2013), have IgY instead of IgG and IgE (Conroy et al. 2014), undergo somatic gene conversion to generate antibody diversity (Warr, Magor, and Higgins 1995), and are missing key immune genes (TNF-α, RIG-I) (Lovell et al. 2014; Barber et al. 2010).

Since the 1950s commercial chickens have been bred for either egg or meat production. This divergence increased production efficiency compared to a dual-purpose breed. Their short generation interval (20-25 weeks) is ideal for making quick genetic progress. Chickens are a valuable source of nutrition around the world, and demand for chicken products will continue to increase as the human population grows. Additionally, compared to other livestock species, chickens are very environmentally friendly (Herrero et al. 2013; Pelletier and Tyedmers 2010). Chicken waste can even be recycled and reused for energy (Petkova et al. 2012).

Chickens in the developing world

In developing countries chickens can be used as a tool to alleviate poverty and promote gender equality (Gueye 2000). Management of backyard poultry by women ensures a reliable protein source for growing children, and empowers women. The commercial broiler and layer industry is less prominent in Africa because the people prefer the taste of eggs and meat from indigenous flocks (Gueye 2000). In village settings, chickens generally scavenge for food; i.e. earthworms, insects, harvest residues, etc. (Gueye 2000). Productivity is low under African village conditions; hens lay between 20 and 100 eggs per year (Gueye 2000). Open production units come with challenges, such as uncontrolled mating and high mortality rates. In uncontrolled mating scenarios the population genetics changes are due to natural selection and genetic drift. Many farmers share a male rooster among neighbors for breeding, which increases inbreeding levels (Dana et al. 2010). The genetic gain that could be attained with artificial selection is unexploited. In a backyard setting, farmers allow more eggs to incubate under the hen due to the high mortality rates that threaten their flock (Gueye

2000). Lowering the mortality rates would lead to more laying hens producing more eggs to be used for consumption or sale.

In some regions of Africa, poultry are used for more than food consumption, but are thought to have mystical properties. Some farmers believe that chickens showing neurological symptoms (such as those caused by viral infection with Newcastle disease) are actually chickens that have protected the family from bad spirits (Gueye 2000). This may delay culling of such birds, allowing the disease to spread, and may dissuade farmers from making proactive decisions to prevent disease. It is predicted that 10-25% of a household's monthly income is at stake under current livestock disease conditions in Madagascar (Rist et al., 2015). A survey of poultry farmers in Ethiopia reported that vaccination services were only available to 5% of the village chickens across all regions (Dana et al. 2010). Diseases like Newcastle disease, that impact the vulnerability of small-scale farmers by causing high levels of mortality, are a particular challenge to poverty reduction (Perry and Sones 2007) and among the biggest fears of most farmers (Perry and Grace 2009).

Newcastle disease

Newcastle disease (ND) is not a new threat to poultry, it was first recognized in 1926 (Afonso et al. 2012). The wide range of clinical signs caused by ND made its identification and characterization difficult, which also resulted in many synonyms: pseudo-fowl pest, pseudovogel-pest, atypische Geflugelpest, pseudo-poultry plague, avian pest, avian distemper, Ranikhet disease, Tetelo disease, Korean fowl plague, and avian pneumoencephalitis (Alexander and Senne 2008).

Nearly 100 years since its discovery, ND still has a significant economic impact on the poultry industry. ND is distributed across the world, North America, South America,

Europe, Asia, South-East Asia, Oceania, and Africa (Palgen et al. 2015). Consistent vaccination and boosting costs the industry a substantial amount of money, and unfortunately many small holder farmers may not be able to utilize this strategy. Outbreaks of “exotic” (velogenic) ND like the 2002 California outbreak, cost $160 million ("Exotic Newcastle disease battle ends" 2003). One reason for the large economic impact of ND is its high prevalence across the world.

Newcastle disease is a result of Newcastle disease virus (NDV). NDV can infect more than 200 bird species (Afonso et al. 2012), and its reservoirs include doves, pigeons, and cormorants (Alexander and Senne 2008). The transmission of the virus is likely aerosol or via the feces of infected birds (Alexander and Senne 2008). It can be spread via the reservoir birds, movement of people and equipment, contaminated feed or water, and through the air (Alexander and Senne 2008). The clinical signs of ND are highly dependent on the infection strain and range from asymptomatic to high mortality (Brown, King, and Seal 1999; Al-Garib, Gielkens, Gruys, and Koch 2003). Virulent strains of ND have a mean death time of approximately 50 hours (Nagai, Klenk, and Rott 1976). Variable clinical signs and widely performed vaccination regimens means diagnosis must be made via real time RT-PCR to detect the RNA genome (Afonso et al. 2012).

NDV belongs to the Mononegavirales order, Paramyxoviridae family, paramyxovirinae subfamily, Avulavirus genus, and Avian Paramyxovirus serotype-1 serotype (Kapczynski, Afonso, and Miller 2013). Other important viruses belonging to the paramyxovirinae subfamily include measles, mumps, Sendai virus, Hendra, Nipah, and parainfluenza viruses (Chang and Dutch 2012). NDV is an enveloped, negative sense, non-segmented, single stranded, RNA virus (Kapczynski, Afonso, and Miller 2013) with rounded,

helical capsid symmetry including ~8 nm projections; in whole the pleomorphic viral particles range from 100-500 nm in diameter (Alexander and Senne 2008).

The NDV genome is approximately 15 kb (always a multiple of six nucleotides) and includes six genes 3'-NP-P-M-F-HN-L-5' (Chambers et al. 1986). NDV encodes its own polymerase, the L protein associates with the nucleocapsid (Alexander and Senne 2008). The nucleocapsid protein (NP) covers the genomic RNA (Yue et al. 2008). The phosphoprotein (P) and L protein are needed for NDV synthesis to occur (Hamaguchi et al. 1983). Through RNA editing mechanisms, the P gene also encodes for two other protein products, the V and W protein (Steward et al. 1993). The V protein is an interferon agonist that promotes the degradation of STAT1 (Parks and Alexander-Miller 2013). Little is known about the function of the W protein, but it is likely not involved in NDV growth or pathogenesis (Huang et al. 2003). The matrix (M) protein helps organize viral proteins at the cell membrane and is required for NDV budding (Pantua et al. 2006). Hemagglutinin-neuraminidase (HN) proteins are found on the surface of NDV particles. Hemagglutinin binds to and neuraminidase is able to cleave sialic acid from the host cell membrane. Along with the fusion (F) protein, HN is critical for the attachment and entry to host cells and may impact virulence (Huang et al. 2003). The F protein is a glycoprotein found on the surface of NDV and is the major determinant of NDV virulence (Panda et al. 2004; Nagai, Klenk, and Rott 1976; Alexander and Senne 2008; Peeters et al. 1999).

The virus can be grouped into 3 categories of virulence: velogenic, mesogenic, lentogenic. Velogenic NDV, or exotic NDV, can be viscerotropic, leading to hemorrhage of the intestine, or neurotropic, which impacts the nervous system (Afonso et al. 2012). Infection with viscerotropic or neurotropic velogenic strains results in acute severe systemic

illness and paralysis, respectively (Brown, King, and Seal 1999). Mesogenic and lentogenic infections usually result in no overt clinical signs, but mesogenic strains do cause more histological lesions than lentogenic strains (Brown, King, and Seal 1999). Lentogenic viruses can range from asymptomatic subclinical enteric infection to mild respiratory infection (Kotani et al. 1987; Ranaware et al. 2016). Lentogenic strains only pose risk to birds with a compromised immune system, and in young birds with no protective maternal antibodies (Afonso et al. 2012). However, lentogenic strains may increase the susceptibility of chicks to secondary infection (Brown, King, and Seal 1999). The viral strains can be classified using Intracerebral Pathogenicity Index (ICPI) scores (Afonso et al. 2012).

As mentioned previously, the major determinant of NDV virulence is the F protein (Panda et al. 2004; Nagai, Klenk, and Rott 1976; Alexander and Senne 2008; Peeters et al. 1999). The uncleaved F protein (F0) must be cleaved into the F1 and F2 protein to have infectious viral progeny (Nagai, Klenk, and Rott 1976; Alexander and Senne 2008; Peeters et al. 1999). The F0 cleavage site in lentogenic viruses is a monobasic amino acid motif, whereas, mesogenic and velogenic strains have a multi-basic amino acid motif (Glickman et al. 1988). The F0 protein from mesogenic and velogenic strains can be cleaved intracellularly by furin-like proteases found in a wide range of host cells (Alexander and Senne 2008). Whereas, for lentogenic strains, trypsin like enzymes are required to cleave the F0 protein extracellularly, these enzymes are found in respiratory and intestinal epithelial cells (Nagai, Klenk, and Rott 1976; Alexander and Senne 2008). In a cell culture absent of trypsin, lentogenic viruses can be rescued by the addition of trypsin (Nagai, Klenk, and Rott 1976).

NDV infection and replication are well characterized. The HN protein mediates the attachment of virus to host cell and the F protein fuses the virus and host cell membranes in a

pH-independent manner (Alexander and Senne 2008; Chang and Dutch 2012). However, cholesterol levels and pH have been shown to impact fusion, which suggests NDV may also infect cells via the caveolae-dependent endocytic pathway (Cantin et al. 2007). Once fusion of the virus and host cell occurs, the nucleocapsid complex is able to enter the cell (Alexander and Senne 2008). The RNA-directed RNA-polymerase (L protein) produces positive strand mRNA from the negative strand genome in the cytoplasm (Alexander and Senne 2008). The mRNA is translated using host machinery (Alexander and Senne 2008). The newly translated F0 protein needs to be cleaved by host proteases in order to be functional (Nagai, Klenk, and Rott 1976; Alexander and Senne 2008). The synthesized viral proteins are transported to the modified regions of the cell membrane for assembly by the M protein prior to budding (Dortmans et al. 2011; Alexander and Senne 2008). The switch from mRNA to genomic production occurs when sufficient amounts of viral proteins have been made (Dortmans et al. 2011). Understanding the viral replication process is important in order to identify steps where the host could intervene.

Host-pathogen interaction

The interaction between virus and host determines the success of infection. A virus that is well suited to its host adapts mechanisms of evading the host immune system. HN and F proteins activate complement and, therefore, paramyxoviruses utilize host mechanisms to inhibit complement by including CD46 and CD55 into the viral envelope (Parks and Alexander-Miller 2013). NDV is sensitive to the antiviral effects of interferon proteins, but the V protein is able to block interferon signaling by phosphorylating STAT1 (Qiu et al. 2016). La Sota NDV has been shown to incorporate up to 30 cellular proteins into its viral particles (Ren et al. 2012). Incorporation of host proteins may aid in evading the host

immune system and increase fusion and replication. On the other hand, host strategies used to defend against the virus may differ between individuals.

To combat the virus, the host utilizes many genes and pathways. N and NP proteins induce autophagy (Cheng et al. 2016), which may increase the frequency of viral protein presentation on MHC class I. Protein kinase R (PKR) phosphorylates eIF2α, which inhibits NDV replication (Zhang et al. 2014). Molecules within the extracellular matrix (collagen and heparin sulfate) limit the spread of NDV (Yaacov et al. 2012). An altered mouse cell line with constitutive IFNα and IFNβ production inhibited the accumulation of viral mRNAs and proteins, were considered resistant to NDV (Aoki et al. 1994).

Whether host responses to velogenic NDV are beneficial or destructive to the host is not always clear. Challenge with velogenic NDV induces a strong iNOS response in the spleen at 3 dpi (Rue et al. 2011). In the bursa, IgM+ cells underwent apoptosis after infection with velogenic NDV (Kristeen-Teo et al. 2017). The cytokine IL2 activates T cells and was therefore used to make a recombinant virulent NDV strain that expressed chicken IL2 (Susta et al. 2015). The recombinant virus decreased viral titers in blood, spleen, oral and cloacal secretions and caused milder lesions, however, the increased IL2 expression did not affect the morality rates (Susta et al. 2015). A strong innate immune response does not prevent disease and death due to velogenic NDV (Rue et al. 2011). The host's cell mediated immune response is important for managing NDV infection, however, cell mediated immune response alone is not protective against velogenic NDV, humoral immunity is required (Reynolds and Maraqa 2000). Chickens are generally unable to survive velogenic NDV without prior vaccination.

Vaccination is currently the best strategy for managing NDV

Lentogenic, mesogenic, and velogenic viruses all belong to the same serotype, which allows the lentogenic strains to be used as a vaccine. The lentogenic La Sota strain is a commonly used vaccine that was isolated from the farm of Adam LaSota in New Jersey, USA in 1946 (Goldhaft 1979). The La Sota strain provided 100% protection against virulent CA/2002 virus (Miller et al. 2013). Increased antibody levels decreased viral shedding and the number of infected birds (Miller et al. 2013). Increased antibody levels can be obtained if time is given for the bird's immune responses to fully develop and if the vaccine and challenge strain are homologous (Miller et al. 2013; Yang et al. 2017). Vaccination to NDV, however, may weaken the immune system and lead to secondary infections. Vaccination programs may fail due to improper dosage or scheduling, not vaccinating the entire flock, early disease outbreaks, and one study suggests that lentogenic strains only partially protect against some velogenic strains (Roohani et al. 2015).

Vaccines are currently the best strategy for managing the impact of NDV, but with many caveats in developing countries (Dimitrov et al. 2017). Vaccines are not available to many farmers with backyard flocks due to distribution and cost (Dana et al. 2010). Also, the non-commercial setting makes a vaccination schedule difficult, plus vaccines must be boosted and reapplied every generation. Moreover, vaccines are not sustainable, other strategies to combat NDV in developing countries must be considered.

Breeding for disease resistance

With proper extension services, breeding for disease resistance may be a useful complementary approach to vaccination in developing countries. Host genetic variation can be utilized for breeding birds that are relatively resistant to NDV. Resistance is defined as the ability of the host to interfere with the pathogen life cycle and can be measured by lower

viral load or greater antibody titers following infection (Bishop 2014). Genetic and breed differences in resistance to NDV were reported as early as 1942 by Albiston and Gorrie who documented mortality rate was higher in the White Leghorns than the Australorps and Rhode Island Reds (Albiston and Gorrie 1942). Again in 1961, mortality differences in response to NDV were observed between sire families, and the authors suggested selective breeding may increase the level of resistance of a flock (Cole and Hutt 1961). Divergent selection of lines based on phytohemagglutinin (an indicator for cell-mediated immunity) impacted the nitric oxide response to NDV (Sundaresan et al. 2005). A genome wide association study (GWAS) identified regions of the genome associated with antibody response to NDV (Luo et al. 2013). One study found associations between single nucleotide polymorphisms (SNPs) in the MHC Class II gene and body weight and NDV viral titer (Molee et al. 2016). Clearly, genetics has an impact on host response to NDV. Genetic selection for disease resistance must be done carefully, because selection on specific immune traits may negatively impact other immune traits, or selection for resistance to one disease can increase susceptibility to another disease (Pinard-van der Laan, Siegel, and Lamont 1998). Thus, before selection for disease resistance is implemented, more information is needed to identify mechanisms of resistance.

Fayoumi and Leghorn

Inbred lines are an excellent research tool. Because immune traits are usually controlled by many genes and strongly impacted by the environment, it is often difficult to detect phenotypic differences within outbred populations. A phenotype is the sum of the genetics and environment. If variation due to genetics is 0, differences in the phenotype between environments (or treatment groups) are caused by the environment. This also allows

smaller differences among treatment groups to be detected. On the other hand, when comparing two inbred lines in the same environment, differences between the lines are a result of their genetic makeup. Two inbred chicken lines that have been well characterized were derived from the Fayoumi and Leghorn breeds. After about 60 years of inbreeding, the homozygosity in the Fayoumi and Leghorn lines was 99.95% and 99.97%, respectively (Fleming et al. 2016).

The Fayoumi and Leghorn inbred lines are maintained at the Iowa State University Poultry Farm (Ames, IA). The Fayoumis have been maintained at Iowa State University since 1954 (Lamont and Chen 1992). They originated from Egypt and were imported to the United States because of anecdotal evidence suggesting greater resistance to avian leukosis (Lamont and Chen 1992). Prior to their arrival at Iowa State, they underwent no artificial selection for production traits, but we assume they have undergone a long history of natural selection for the harsh environment in Egypt. The original Leghorn (GH) line was established at Iowa State University in 1954 from the descendants of a Ghostley Hatchery dam and a Heisdorf and Nelson sire (Lamont and Chen 1992). A subline of GH, the GHs, was brought back to Iowa State University after some absence and re-derived in 1965 (Lamont and Chen 1992). Derived from commercial lines, the Leghorns have a history of selection for egg production traits.

Although the Fayoumi and Leghorn inbred lines are phylogenetically distinct (Zhou and Lamont 1999), there is no significant difference in body weight between the Fayoumis and Leghorns from hatch to 8 weeks of age (Deeb and Lamont 2003). The average generation intervals in the inbred lines is 9-10 months (Zhou and Lamont 1999), suggesting that both lines have undergone about 80 generations of inbreeding to date. The only selection

on these inbred lines is indirect selection for reproduction and survivability, which has occurred in both lines. Blood-typing of the breeders occurs every generation to ensure the correct MHC haplotype is present and to prevent pedigree errors.

The Fayoumi and Leghorn inbred lines have been included in many studies during their tenure at Iowa State University. Most studies focused on the immune system of the two lines, but even their breast meat quality and composition have been compared (Lonergan et al. 2003). Also, differences in the expression of adipogenic genes in adipose tissues were found between the Fayoumis and Leghorns, and Fayoumis had larger adipocyte volume (Ji et al. 2014).

Many differences in the genetic material of the Fayoumi and Leghorn have been proposed to contribute to their different immune phenotypes. Lung tissue from the Fayoumi and Leghorn lines showed differential methylation in regions of the genome associated with immune gene-ontology (GO) enrichment terms including immunoglobulin domain, immune effector process, and leukocyte mediated immunity (Li et al. 2015). This suggests methylation patterns may contribute to differences in host response seen between these two lines. The Fayoumi and Leghorn lines also have different endogenous viral (ev) genes and selection for immune related traits impact the frequency of ev loci (Lamont and Chen 1992), suggesting that differences in the ev genes may contribute to the phenotypes of the Fayoumi and Leghorn lines. Copy number variants in the Fayoumis and Leghorns have been related to immune system pathways and GO terms, and many are found on chromosome 16, the site of the polymorphic major histocompatibility (MHC) region (Abernathy et al. 2014). The Fayoumis have two copies of defensin7, however, mRNA expression level in the Fayoumis was not greater compared to the Leghorns (Lee et al. 2016). In offspring from an F_2 cross

between the Fayoumi and Leghorn lines, antibody levels in response to the *Salmonella enteritidis* (SE) vaccine were greater in birds that had inherited the Fayoumi IFN-γ promoter compared to those with the Leghorn version (Zhou, Lillehoj, and Lamont 2002). SNP analysis of the NK-lysin gene showed that the allele that conferred more antimicrobial activity was present in the Leghorn line and not in the Fayoumi line (Lee et al. 2014). Thus, the Fayoumi and Leghorn lines have several differences in their DNA that influence the immune system.

The mRNA expression levels of immune genes are important in predicting how the immune system responds to specific stimuli. The mRNA expression levels of Toll like receptor (TLR) genes were compared in the Fayoumi and Leghorn lines after challenge with *Salmonella* (Abasht et al. 2009). TLR proteins identify pathogen associated molecular markers, and are very important for innate immunity. The Fayoumi expressed significantly higher TLR2 and TLR4 mRNA than the Leghorn (Abasht et al. 2009). In peripheral blood mononuclear cells (PBMCs), there was no significant difference in the expression of IL2, IL6, CXCLi2, or TGF-β4 mRNA between the adult Fayoumis and Leghorns after exposure to SE (Kaiser et al. 2006). The Leghorn had greater expression levels of CXCLi2, CCLi2, and IL12α mRNA than the Fayoumi in the spleen of day-old chicks challenged with SE (Cheeseman et al. 2007). Leghorn heterophils expressed higher levels of CXCLi2 mRNA after stimulation with SE than the Fayoumis, resulting in higher levels of inflammation, which can be damaging (Redmond et al. 2011). Unfortunately, the use of quantitative PCR (qPCR) as these studies did, limited the number of genes that could be efficiently examined.

In vitro assays offer important information on cell function and activity, which may be due to genetics and gene expression. In peripheral blood leukocytes under resting

conditions, Leghorns had significantly higher surface expression of CD3, but fewer CD3+ cells than the Fayoumis, as measured by flow cytometry (Cheeseman, Kaiser, and Lamont 2004). Bone marrow derived dendritic cells (BMDC) from the Fayoumis and Leghorns were characterized in response to lipopolysaccharide (Van Goor et al. 2016). Fayoumi BMDC had greater MHC II surface expression, higher phagocytic ability, and after LPS treatment produced more nitric oxide than the Leghorns (Van Goor et al. 2016). Heterophils, the chicken version of neutrophils, are very important in the innate immune response. *In vitro*, Fayoumi heterophils had significantly more heterophil extracellular traps-DNA release, but lower phagocytosis and bacterial killing than the Leghorn heterophils (Chuammitri et al. 2011). Fayoumi heterophils utilize extracellular killing mechanisms, whereas the Leghorn heterophils favor intracellular defense mechanisms. After treatment with corticosterone (to mimic stress), the heterophil to lymphocyte (H/L) ratio was increased for Leghorns, but did not change for Fayoumis, suggesting a higher tolerance to stress-induced immune suppression in the Fayoumi (Redmond et al. 2011). The Fayoumi and Leghorn have shown distinct responses to many pathogens and stimulants.

Overall, evidence suggests that the Fayoumis are more resistant to many pathogens when compared to the Leghorns. After challenge with low-path avian influenza virus (AIV), the Fayoumis had lower viral load than the Leghorns (Wang et al. 2014). Less tumors and clinical signs in response to Marek's disease virus (MDV) also suggest the Fayoumis are more resistant compared to the Leghorns (Lakshmanan, Kaiser, and Lamont 1996). In addition, a different Fayoumi line had less mortality and higher weight gain than a White Leghorn line after infection with *Eimeria tenella* (Pinard-van der Laan et al. 2009). Observations from the Iowa State Poultry Farm showed higher mortality rates in response to

the NDV/Infectious Bronchitis vaccine in the Leghorns than Fayoumis (Lamont, personal communication). It is unknown how these lines will respond to NDV alone, but we predict the Fayoumis will also be relatively resistant to NDV. Comparing the Fayoumi and Leghorn's response to NDV can identify potential mechanisms of resistance.

RNA-seq

The transcriptome is an intermediary phenotype that links the genome to the proteome. RNA-sequencing (RNA-seq) is a state of the art technology that has revolutionized RNA biology over the past decade. Researchers no longer have to create primers or probes to examine the expression levels of genes of interest; RNA-seq measures all of the mRNA within a tissue or cell. Now, with the high-throughput capability of RNA-seq, the transcriptomes of hundreds of individuals can be sequenced at once. RNA-seq has several different applications including, differential expression, relative expression, alternative splicing, transcript and isoform discovery, RNA editing, allele-specific expression, gene fusion discovery, and eQTL analyses (Han et al. 2015). The relative ease of studying mRNA compared to protein makes expression studies so common.

The basic steps in an RNA-seq experiment are outlined here. High quality total RNA is isolated from a tissue sample and DNase treated to remove any genomic DNA. Isolated RNA is then converted into cDNA libraries. Library preparation is highly dependent on the kit and sequencing platform used. Estimates of cost, yields, run times, error rates, etc. between different sequencing machines have been reported (Han et al. 2015). The Illumina protocol involves purifying and fragmenting the mRNA, cDNA synthesis, ligation of adaptors (allows for multiplexing), and PCR amplification. Libraries are then validated, normalized for concentration, pooled, and added to a lane within the flow-cell, where the

samples are sequenced. There is minimal variation due to technical differences such as lane and flow cell, but slightly larger technical variation was observed when comparing library preparations (Bullard et al. 2010). Adapters bind to oligos on the flow cell and undergo an amplification step to form clusters of double-stranded cDNA. In the sequencing machine, fluorescently labeled nucleotides are added in cycles. Their fluorescent signal records the nucleotide that was added. The number of cycles equals the sequence read length. Reads from each sample must undergo quality filtering, mapping to the genome, and counting of the number of reads to a genomic feature. Multiple programs and packages are available for each of these steps, some of which have been previously described (Han et al. 2015). The expression level for each gene is determined by the number of sequenced reads (counts) that map to that gene.

Normalization of counts prior to analysis is critical to account for different sequencing depths between libraries, and this step can have a large impact on a differential expression analysis (Bullard et al. 2010). Differential expression analysis identifies genes or transcripts that have statistically different counts between treatment groups. DESeq2 and edgeR are commonly used programs to test for differential expression, and both have been used in this dissertation. The software edgeR uses trimmed mean normalization and DESeq uses a relative log expression normalization approach (each library is compared against a virtual library) (Anders et al. 2013). To estimate dispersion, edgeR shrinks the feature-level dispersion estimates towards the trended mean based on the dispersion-mean relationship (Anders et al. 2013), whereas DESeq uses the maximum dispersion estimate and the dispersion-mean trend (Anders et al. 2013). These differences make edgeR more sensitive to outliers and give DESeq less power (Anders et al. 2013). A modified Fisher exact test

assuming a Negative Binomial distribution is used in both edgeR and DESeq to calculate p-values for each gene (Rapaport et al. 2013).

Unlike differential expression analyses, co-expression analysis does not require contrasts and can be used to correlate expression values with phenotypes or factors of interest. The clustering of genes based on their expression patterns based on a co-expression network analysis can be used to identify novel or known genes and suggest their relationship with a particular biological process or function, depending on the design of the experiment (Serin et al. 2016). Co-expression analyses are particularly useful when few genes are identified as differentially expressed or when dealing with a particularly complex experimental design. The software Weighted Gene Correlation Network Analysis (WGCNA) (Langfelder and Horvath 2008) was used for co-expression analysis in this dissertation. WGCNA clusters genes into modules based on their co-expression and correlates those modules with treatment groups or phenotypes of interest (Langfelder and Horvath 2008). Modules of interest must undergo further analysis to determine the function of the genes within the modules.

NDV impacts host gene expression

The level of expression of a gene under a specific condition is a highly heritable trait that can also greatly impact an animal's response to disease (Dermitzakis 2008). Many studies have examined the host response to NDV in order to better understand the impact of NDV on gene expression in multiple tissues. Examining the mRNA expression of key immune genes is often done with qPCR. In egg laying hens challenged with velogenic NDV, qPCR showed TLR3/7/21, MDA5, IL2, IL6, IL1β, IFNβ, CXCLi1, CXCLi2, and CCR5 were upregulated in the magnum and uterus (Li et al. 2016). The spleen showed CCLi3,

CXCLi1, CXCLi2, IFNγ, IL12α, IL18, IL1β, IL6, iNOS, TLR7, MHCI, IL17F, and TNFSF13B were upregulated after challenge with velogenic NDV as measured by qPCR (Rasoli et al. 2014). Velogenic NDV induces a strong innate immune response. Different NDV strains result in different host cytokine expression patterns (Liu et al. 2012).

Microarray analysis was a popular method used prior to the advent of RNA-seq that allowed high-throughput analysis of mRNA expression, but it has many pitfalls compared to RNA-seq such as problems with fluorescence intensity limits and the limited probes included in the array. Microarray analysis performed on spleens of chickens infected with two strains of velogenic NDV showed varying expression levels in inflammatory related genes; these differences corresponded to the divergent gross pathology observed in the birds (Hu et al. 2014). Both velogenic strains upregulated MDA5 and Mx and predicted the enrichment of inflammatory response, cell death, and immune cell trafficking in the spleen (Hu et al. 2014). After challenge with a velogenic strain of NDV, the spleens from specific-pathogen-free chickens were analyzed using microarray at 1 and 2 dpi (Rue et al. 2011). Several immune related genes were upregulated due to challenge, including IL6, MIP3α, Mx, IFIT5, ISG, and MDA5 at 1 dpi and iNOS, IL1β, IL18, IL8, and IFNγ at 2 dpi (Rue et al. 2011). Primary chicken embryo cells downregulated interferon and cytoskeletal related genes after infection with velogenic NDV (Munir, Sharma, and Kapur 2005). Infection strain, tissue, dpi, genetic line, all can impact the findings of an expression study.

Tracheal epithelial cells from several indigenous African chicken breeds were cultured and infected with NDV (Ommeh 2013). The differentially expressed genes (DEG) detected by RNA-seq predicted a strong innate immune response (Ommeh 2013). Chicken induced pluripotent stem cells were selected for tolerance to La Sota NDV for 9 generations,

and after selection the 36 times more tolerant cells were still permissive to infection with velogenic and lentogenic NDV (Susta et al. 2016). RNA-seq analysis found 1,009 DEG between the unselected cells and the cells after 9 rounds of selection that were predicted to impact endocytosis, mTOR signaling pathway, apoptosis, WNT signaling pathway, neuroactive ligand-receptor interaction, protein processing in endoplasmic reticulum, regulation of actin cytoskeleton, and cell adhesion molecules (Susta et al. 2016). These pathways may have a role in tolerance to NDV. Although RNA-seq is commonly used in chickens, to the best of the author's knowledge, prior to the experiments discussed in this dissertation no studies have used RNA-seq to study the host response to lentogenic NDV *in vivo*.

Tissue choice

Gene expression studies are useful only if they are performed in biologically relevant tissues or cell types (Dermitzakis 2008). When studying host response to lentogenic NDV, it is relevant to examine tissues near the sites of inoculation. After infection with lowly virulent NDV, histologic lesions were only observed in respiratory tissues and at the inoculation site (Kommers et al. 2003). The vaccine strain, La Sota is only able to replicate near the site of inoculation (Wakamatsu et al. 2006). Air sac infection was consistently found after chickens were challenged with velogenic, mesogenic, or lentogenic NDV strains (Brown, King, and Seal 1999). In this dissertation, the high-titered La Sota challenge virus was administered via oculonasal routes. Therefore, tracheal epithelial cells, lung, and Harderian gland were optimal tissues due to their proximity to the site of infection and immune capabilities.

The trachea is a membranous tube that connects the larynx to the bronchial tubes of the lungs; its major function is to transport air to and from the lungs. Due to its function and

location the trachea also plays a crucial role in the host immune response. Pathogens that enter the body through the air are first detected here. The cleansing function of the mucociliary system clears foreign antigens (Kotani et al. 1987).

The trachea's response to NDV has been well characterized. Virus was detected in the trachea epithelium 24 hours after exposure to both lentogenic and velogenic NDV strains (Beard and Easterday 1967). The viral titers seen in the trachea showed comparable patterns in the B-1 (lentogenic) and GB (velogenic) strains over time only when the route of infection was aerosol (Beard and Easterday 1967). In the trachea, tissue damage was highest when viral antigen was detectable, but the repair process began once antibodies were produced (Kotani et al. 1987; Mast et al. 2005). After inoculation with La Sota NDV, IgM and IgA were detected in trachea washes at 4 dpi and IgG was detected by 7 dpi (Al-Garib, Gielkens, Gruys, Hartog, et al. 2003).

Many studies have observed the histological impact of aerosol exposure of NDV on the trachea (Beard and Easterday 1967; Bang, Foard, and Bang 1974; Kotani et al. 1987; Mast et al. 2005). Increased mucous levels were observed in the trachea from 1 to 8 dpi with a lentogenic NDV strain (Kotani et al. 1987). Thorough descriptions of the histological changes occurring in the trachea after aerosol exposure to lentogenic NDV in 40 day-old SPF chickens from 1-24 dpi have been described previously (Kotani et al. 1987) and include deciliation of the epithelium, goblet cell hypertrophy, edema, and heterophil, lymphocyte, and plasma cell infiltration (Kotani et al. 1987). In addition, engorged capillaries have been reported (Beard and Easterday 1967). La Sota NDV causes major changes in the trachea (Mast et al. 2005), which will also likely impact the gene expression.

The tracheal epithelial cells' responses to both AIV and NDV have been previously analyzed using RNA-seq. The targets of miRNAs differentially expressed in the trachea between the birds challenged with AIV and nonchallenged birds showed largest enrichment of the interleukin-12 production and interleukin-12 biosynthetic process GO term (Wang et al. 2009). Differentially expressed genes showed strong innate immune responses in tracheal epithelial cells infected with NDV *in vitro* (Ommeh 2013). Further characterization of this tissue's response to NDV *in vivo* is necessary.

Avian and mammalian lungs have anatomical differences that may be related to the evolution of flight. Unlike mammals, avian lungs are incised by the dorsal rib cage (Duncker 1972), their volume does not change with respiration phase (Duncker 1972), and they are relatively smaller than mammalian lungs (de Geus, Rebel, and Vervelde 2012). Although the primary function of the lungs is gas exchange, they also play an important role protecting the host from respiratory pathogens. The lung contains many immune cells both distributed throughout the tissue and highly organized into bronchus-associated lymphoid tissue (BALT) (de Geus, Rebel, and Vervelde 2012). BALT does not need to be induced; it is a normal lung component that helps compensate for the missing lymph node system (Fagerland and Arp 1993). By 3 to 4 weeks of age BALT structures can be found in the chicken lung (Fagerland and Arp 1993). After antigen uptake, antigen-presenting cells migrate to BALT (de Geus, Rebel, and Vervelde 2012). The function of the lung in response to NDV has not been well characterized, however, it is known that avirulent NDV strains that are passaged through chicken air sacs and embryos have the ability to become virulent (Meng et al. 2015).

Previously gene expression studies of the lung have examined host response to AIV. A microarray study showed distinct responses to low and high path AIV in the chicken lung

(Ranaware et al. 2016). The targets of miRNAs differentially expressed in the lung due to challenge with AIV showed significant enrichment of response to virus, immune system response, lymphocytes, and lung development GO terms (Wang et al. 2009). Both Fayoumis and Leghorns downregulated MHC class I related genes in the lung after challenge with AIV (Wang et al. 2014). The lung's response to NDV has not previously been characterized using RNA-seq.

The function of the Harderian gland is not well characterized, but is discussed in more detail in chapter 4 and appendix I. The Harderian gland is located on the underside of the orbit behind each eyeball, is brownish red in color, irregularly shaped, and weighs approximately 85 mg in adult chickens (Wight et al. 1971). The Harderian gland lubricates the nictating membranes (Bang and Bang 1968) and cleans and lubricates the cornea (Wight et al. 1971). The Harderian gland is also known to play a role in local immune response (Burns 1976). Early studies showed the Harderian gland was composed of large numbers of plasma cells and heterophils (Bang and Bang 1968), and the number of plasma cells increased with age (Wight et al. 1971; Davelaar and Kouwenhoven 1976). A more recent study found that the number of T lymphocytes increased with age and the number of plasma cells within the Harderian gland remained constant (Maslak 1994). The appearance of definite germinal centers increased with age (Mueller, Sato, and Glick 1971). IgA produced in the Harderian gland can be secreted via tears (Russell and Koch 1993). The Harderian gland is a unique immune tissue. When stimulated with the same mitogens, the Harderian gland lymphocytes were less responsive compared to the spleen and blood lymphocytes (Maslak 1994).

Because eye-drop and aerosol application of vaccines are popular, understanding the Harderian gland's response is critical. Vaccination for infectious bronchitis virus caused greater vascularization of the Harderian gland (Davelaar and Kouwenhoven 1976). Challenge with lentogenic NDV induced an inflammatory response and increased heterophils in the Harderian gland (Survashe, Aitken, and Powell 1979). Compared to a control group, T cell concentration in the Harderian gland was significantly increased after challenge with lentogenic NDV, and was further increased in birds challenged with velogenic NDV (Maslak 1994). CD8+ cells in particular are predicted to play an important role in the Harderian gland's response to NDV vaccination (Russell, Dwivedi, and Davison 1997). Unfortunately, most studies regarding to the Harderian gland were conducted before many modern techniques were available. Much is left to learn about this tissue that has not previously been analyzed with RNA-seq.

The trachea, lung, and Harderian gland are excellent tissues to examine host response to lentogenic NDV and have been reviewed previously (de Geus, Rebel, and Vervelde 2012). These three tissues are physically linked, as secretions from the Harderian gland drain into the upper respiratory tract (Dohms, Lee, and Rosenberger 1981). Furthermore, viral replication has been detected in the trachea, lung, and Harderian gland after infection with lentogenic NDV (Russell and Koch 1993). When studying mechanisms of host resistance, it is ideal to do so in tissues that directly interact with the replicating virus. Lymphoid tissue is constitutively present in the lung and Harderian gland, but not the trachea (de Geus, Rebel, and Vervelde 2012). Therefore, we expect to see large infiltration of immune cells to the trachea. Analyzing all three tissues will provide a comprehensive picture of the chicken's response to NDV.

Thesis Objectives

This dissertation characterizes the response of the Fayoumi and Leghorn inbred lines to NDV by analyzing the transcriptomes of the tracheal epithelial cells, lung, and Harderian gland in response to lentogenic NDV. Response phenotypes after lentogenic challenge (viral load and antibody levels) and velogenic challenge (mean survival time) will be used to label the Fayoumi and Leghorn as relatively resistant or susceptible to NDV. Using the Fayoumi and Leghorn to model relative resistance and susceptibility, respectively, will enable the identification of genes and pathways related to NDV resistance. Additionally, these results will shed light on the impact of genetics on response to vaccine.

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CHAPTER 2. NOVEL MECHANISMS REVEALED IN THE TRACHEA TRANSCRIPTOME OF RESISTANT AND SUSCEPTIBLE CHICKEN LINES FOLLOWING INFECTION WITH NEWCASTLE DISEASE VIRUS

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Abstract

Newcastle disease virus (NDV) has a devastating impact on poultry production in developing countries. This study examined the transcriptome of tracheal epithelial cells from two inbred chicken lines that differ in NDV susceptibility after challenge with a high-titer inoculum of lentogenic NDV. The Fayoumi line had a significantly lower NDV load post-challenge relative to the Leghorn line, demonstrating the Fayoumi's classification as a relatively NDV resistant breed. Examining the trachea transcriptome showed a large increase in immune cell infiltration in the trachea in both lines at all times post-infection. Pathways conserved across lines and at all three time points post-infection included: iCOS-iCOSL signaling in T helper cells, NF-Kβ signaling, role of NFAT in regulation of the immune response, calcium-induced T lymphocyte apoptosis, phospholipase C signaling, and CD28 signaling in T helper cells. Although there were shared pathways seen in the Fayoumi and Leghorn lines, each line showed unique responses as well. The down-regulation of collagen

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and the activation of EIF2 signaling in the Fayoumis relative to the Leghorns at 2 days post infection may contribute to the resistance phenotype seen in the Fayoumis. This study provided further understanding of host-pathogen interactions, which could improve vaccine efficacy and in combination with genome wide association studies, has the potential to advance strategies for breeding chickens with enhanced resistance to NDV.

Introduction

Newcastle disease is caused by virulent strains of Newcastle disease virus (NDV), a single-stranded, negative sense, non-segmented paramyxovirus that negatively impacts poultry meat and egg production throughout the world. In developing countries, vaccination is sometimes not a viable option, due to lack of infrastructure (cold-chain and transportation) hampering the availability of vaccine, lack of technically trained personnel, the cost of the vaccine, and the need for re-administration. Other solutions must be found in order to control this disease (1). In these countries, virulent NDV can cause mortality as high as 80% among village flocks (2). Ameliorating the negative impacts of NDV would reduce hunger, strengthen food security, alleviate poverty, and since chickens are traditionally a woman's responsibility, promote gender equality in developing countries (3).

In developed countries NDV also has the potential to cause severe damage to the poultry industry, from direct and indirect losses due to trade embargoes and restrictions during outbreaks. Hitchner (4) refers to NDV as a "sleeping giant" that requires close monitoring because the large range of clinical signs and genomic diversity make NDV detection and diagnosis challenging (5, 6), the worldwide distribution increases the potential danger of NDV (5), and although uncommon, viruses of low virulence have the ability to mutate and increase in virulence (7).

The range of NDV clinical signs varies depending on route of infection, environmental factors, host immunity, and strain of the virus (8). The strains of NDV are divided into three major categories from low to high virulence: lentogenic, mesogenic, velogenic (8). The intra cerebral pathogenicity index (ICPI) is used to differentiate and classify NDV according to its virulence (8), which is largely determined by the F protein cleavage site (9). Although the strains differ in virulence, all belong to the same serotype; thus enabling the use of lentogenic viruses as vaccines for protection against the more virulent strains. Although rare, clinical signs associated with infection by lentogenic strains are non-specific such as ruffled feathers, anorexia, decreased egg production, and respiratory infection in birds with a compromised immune system (5, 6, 10).

Understanding host response to NDV is necessary in order to generate improved solutions to combat this devastating disease. Examining the host mRNA expression after challenge provides insight into host-pathogen interaction. Previous studies have compared gene expression levels measured from qPCR and microarray data in response to infection with multiple NDV strains and host tissues and reported different cytokine expression levels dependent on the infection strain (11-15). Gene expression analyses performed on tracheal epithelial cells following NDV infection *in vitro* showed a strong innate immune response (16). The trachea is one of the first tissues NDV will encounter via aerosol transmission, and a critical site of host-pathogen interaction. All strains of NDV are able to replicate in the epithelial cells of the trachea (17), and challenges with either lentogenic and velogenic strains resulted in comparable viral titers in trachea (18).

Although lentogenic NDV generally does not cause severe clinical signs, gene expression changes due to inflammation, tissue destruction, cell proliferation, and tissue

remodeling are expected in the trachea after intranasal inoculation (19). Aerosol delivery can result in deciliation, congestion, goblet cell hyperplasia, edema, and infiltration of heterophils, lymphocytes, and plasma cells in the tracheal mucosa (20, 21). If seeking mechanisms of host resistance, the trachea is an ideal tissue to examine, as this is one of the initial sites of interaction with the virus.

Host genetics may play a large role in host-pathogen interaction. Previous studies have shown that resistance to NDV has a genetic basis (22-25). In the current study two diverse, inbred chicken lines, the Fayoumi and Leghorn, were utilized to identify genetic mechanisms of resistance to NDV. Several studies have compared the immune response of these two lines. Fayoumi were found to be relatively resistant to *Salmonella*, *Eimeria*, Marek's disease, and Avian Influenza compared to the Leghorns (26-29). Bone marrow derived dendritic cells from these two lines showed increased phagocytic ability, nitric oxide production, and MHC-II surface expression (30). Here Fayoumi and Leghorn lines are used as a discovery platform to identify mechanisms of resistance to NDV. We hypothesize that the Fayoumis will be more resistant to NDV, as shown by less viral load and higher antibody titer. The term resistance as used in the current study is defined as the ability of the host to interfere with the pathogen life-cycle (31). The term resistance in this context is not absolute, therefore, the host may still become infected and succumb to virulent NDV strains. Comparing the two lines' response to NDV may designate genes/pathways associated with the Fayoumi's resistance. The information generated by this study will be beneficial for vaccine development and other control strategies.

Results

The Fayoumi's resistant phenotype was upheld by NDV viral, antibody quantification measurements, and sequence data.

To examine the effects of an NDV challenge on the two inbred lines of differing resistance, the challenged Fayoumis and Leghorns were inoculated with the La Sota NDV strain, while the nonchallenged were given saline solution as a mock infection. Lachrymal fluid was collected for viral quantification from each chicken prior to infection, at 2 days post infection (dpi), and at 6 dpi. No NDV was detected in all birds prior to challenge and in all nonchallenged birds at all times (data not shown). In the challenged birds, viral load significantly decreased from 2 to 6 dpi ($p<0.0001$) and line had a significant effect on viral load ($p=0.045$) (Figure 2-1). At 6 dpi the Fayoumis had significantly less virus than the Leghorns ($p=0.0122$), suggesting the Fayoumis may have cleared the virus more quickly (Figure 2-1). There was no correlation ($r=-0.0097$) between each individual's viral load at 2 and 6 dpi, which was in agreement with previous studies (19).

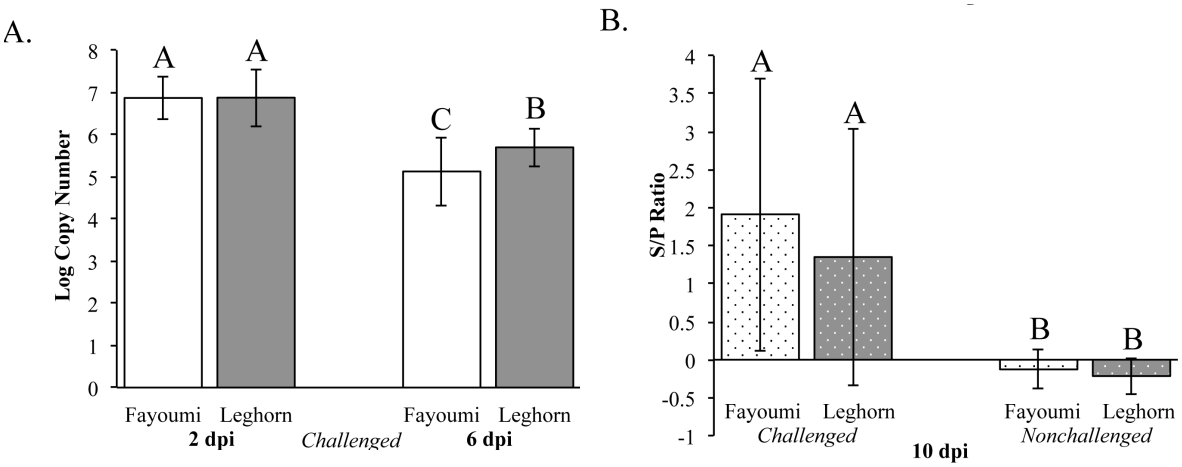

Figure 2-1: Viral and antibody quantification by genetic line and days post infection (dpi). A. Viral load is shown as the LSMeans of log copy number, measured by qPCR, of the Fayoumis (white) and Leghorns (gray). Error bars represent the standard deviation. A Student's t-test was used to derive the connecting letters report in which bars that are not labeled with the same letter are significantly different ($p<0.05$). At 2 dpi Fayoumi n=21, Leghorn n=28, and at 6 dpi Fayoumi n=12 and Leghorn n= 20, total n=81. B. Antibody titer is displayed as LSMeans of the S/P ratio in the Fayoumis (white) and Leghorns (gray) at 10 dpi and the error bars represent the standard deviations. Connecting letters report generated by Students t-test. At 10 dpi, challenged Fayoumi n=8, challenged Leghorn n=13, nonchallenged Fayoumi n=6, and nonchallenged Leghorn n=8, total n=35.

Serum from blood collected at 10 dpi was used to quantify NDV-specific antibody via ELISA. The ELISA S/P ratios at 10 dpi were significantly different (p=0.0007) between the challenged and nonchallenged birds. Due to the large variation in NDV antibody levels, there was no significant difference between the lines within the same treatment category. However, challenged Fayoumis produced more antibodies on average (p=0.367) than the Leghorns (Figure 2-1).

Sequence reads that did not map to the chicken genome were analyzed to determine if any mapped to genes in the NDV genome (Figure 2-2). Viral transcripts were only detectable at 2 dpi in the challenged birds. The main effect of line had a statistically significant impact (p=0.0264) on the viral transcripts of the 2 dpi challenged Fayoumis and Leghorns (Figure 2-2). As expected, the counts per million (cpm) appears higher for genes at the beginning of the virus in both lines.

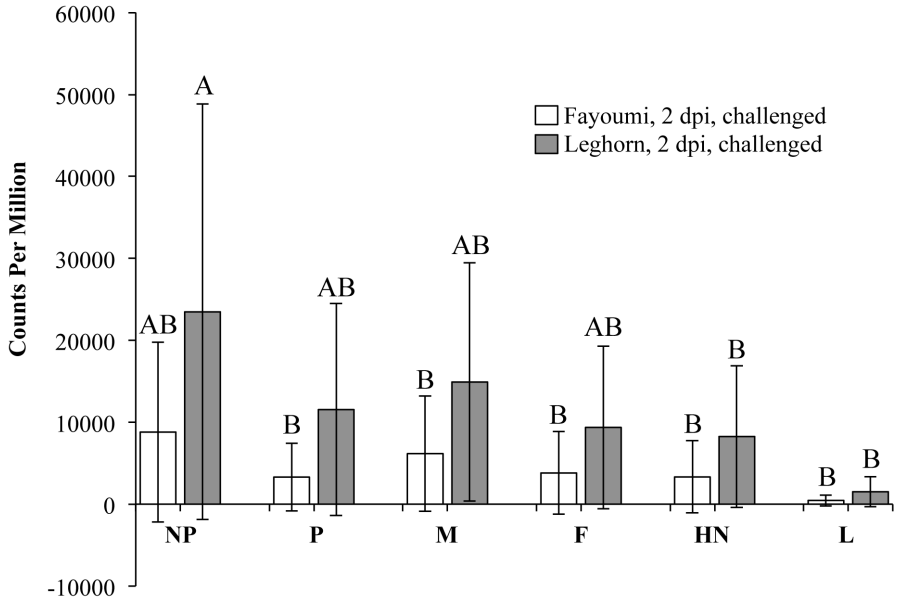

Figure 2-2: The counts per million aligned to each viral gene in the challenged Fayoumi and Leghorns at 2 dpi. The NDV genes listed in order nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large polymerase protein (L). The LS Means of the counts per million are shown for the challenged Fayoumis (white, n=4) and Leghorns (gray, n=5). Error bars represent standard deviation of the mean. Connecting letters report was generated with a Student's T-test.

Effects of NDV challenge on the trachea transcriptome.

Epithelial cells were peeled from the trachea of challenged and nonchallenged birds from both lines at all three times and used for RNA isolation, cDNA library construction, and sequencing. The tracheal epithelium sequencing results were similar across treatment groups and therefore we did not expect biases due to differences in number of reads, mapping percentage, or transcriptome coverage percentage amongst treatment groups (Table 2-1). On average, approximately two million reads were removed from each sample in the filtering process, nearly 90% of the filtered reads were mappable, and about 75% of the Galgal4 transcripts had at least one mapped read (Table 2-1).

Table 2-1: Sequence processing summary averages[a]

Group	Avg. Reads Pre-filter	Avg. Reads Post-filter	Avg. Mapping %[b]	Avg. Transcriptome Coverage %[c]
Challenged	14,351,100	11,019,846	89.7	76.0
Nonchallenged	14,478,105	11,909,690	89.8	75.7
Fayoumi	13,487,623	11,122,267	89.8	76.0
Leghorn	14,313,716	11,770,191	89.8	75.7
Overall	13,900,670	11,446,229	89.8	75.8

[a]Averages (Avg.) were taken across four treatment groups.
[b]The mapping percentage was calculated as the number of reads mapped to the reference genome divided by reads post-filter.
[c]Transcriptome coverage percentage was calculated as the number of transcripts with at least one mapped read divided by the total number of transcripts.

To compare the host response to NDV, the challenged and nonchallenged birds were contrasted within line at each time point in the experiment. These comparisons are referred to as the six major contrasts. As time progressed, there were fewer differentially expressed genes (DEG) between the challenged and nonchallenged birds in both lines (Figure 2-3). Overall, more genes were up than downregulated (Figure 2-3). Differentially expressed genes that were shared between lines are likely crucial to response to NDV for all chickens, whereas genes uniquely identified as differentially expressed in the Leghorn or the Fayoumi

may be related to the susceptibility and resistance, respectively, of the chickens. Protein kinase C beta (PRKCβ) was the only gene that was uniquely identified as DE in the Fayoumi only at all three time points. The large difference in numbers of DEG at 6 dpi in the Fayoumi and Leghorn lines (Figure 2-3) suggests that the Fayoumi recovered more quickly from the virus, resulting in fewer differences between the challenged and nonchallenged birds.

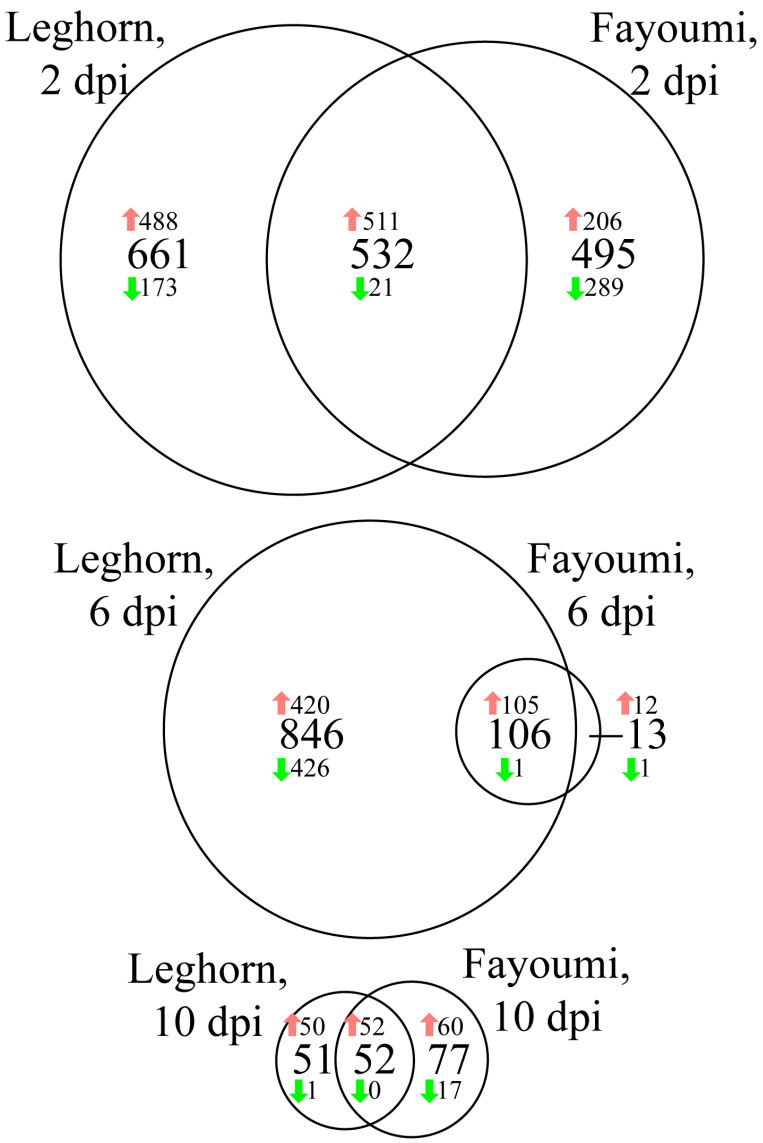

Figure 2-3: Numbers of differentially expressed genes between challenged and nonchallenged birds within each genetic line at three time points. FDR<0.05 was used to classify genes as differentially expressed. Within each portion of the Venn diagram, the number of genes that were upregulated or downregulated in the challenged birds relative to the nonchallenged birds are denoted with red or green arrows, respectively.

Challenge with NDV stimulated infiltration and migration of immune cells.

The DEG from the challenged vs. nonchallenged birds at each time point and within each line were used for cell type enrichment analysis. At 2 dpi, the top cell types enriched were similar for both lines (Figure 2-4); whole blood was the most enriched cell type for both lines. At 6 dpi, the lines were the most dissimilar, which corresponds to the difference in DEG numbers (Figure 2-3 & Figure 2-4). The enrichment of cancer type cell lines at 6 dpi may be a result of high levels of cell proliferation. Overall, the results suggested a conserved response between the two lines and a strong enrichment of immune related cells after challenge at all time points but no clear shifts from innate to adaptive immune cell types over time.

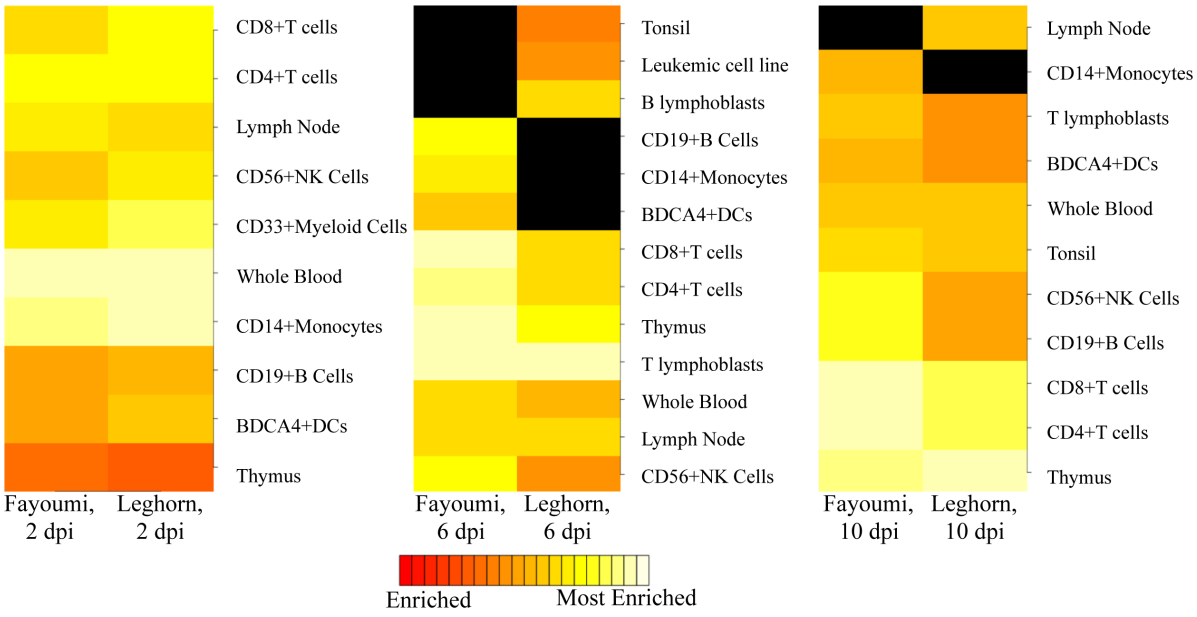

Figure 2-4: Cell type enrichment analysis predicts enriched cell types from DEGs between challenged and nonchallenged birds within each line at each time. The top ten most significantly enriched cell types (listed to the right of each heat map) range from enriched (red) to most enriched (white) in either the Fayoumi or Leghorn, grouped by time. A black fill indicates a cell type that does not fall into the top ten enriched cell types of one line but does in the other. Abbreviations: DCs=dendritic cells; Leukemic cell line=Leukemia Promyelotic HL-60; B lymphoblasts=Lymphoma Burkitts (Daudi); T lymphoblasts=Leukemia Lymphoblastic (MOLT-4)

A major pathway predicted to be activated in all six major contrasts by pathway analysis using IPA® was the leukocyte extravasation signaling pathway. Figure 2-5A shows the genes in this pathway that were significantly up or downregulated within each contrast. Based on expression levels of the genes in this pathway (Figure 2-5B), the Molecule Activity Prediction function of IPA® predicted, for each contrast, activation of cell mobility and activation of actin cytoskeleton contraction; IPA® output places more confidence in the prediction of the latter in the Fayoumis than the Leghorns at all time points (not shown).

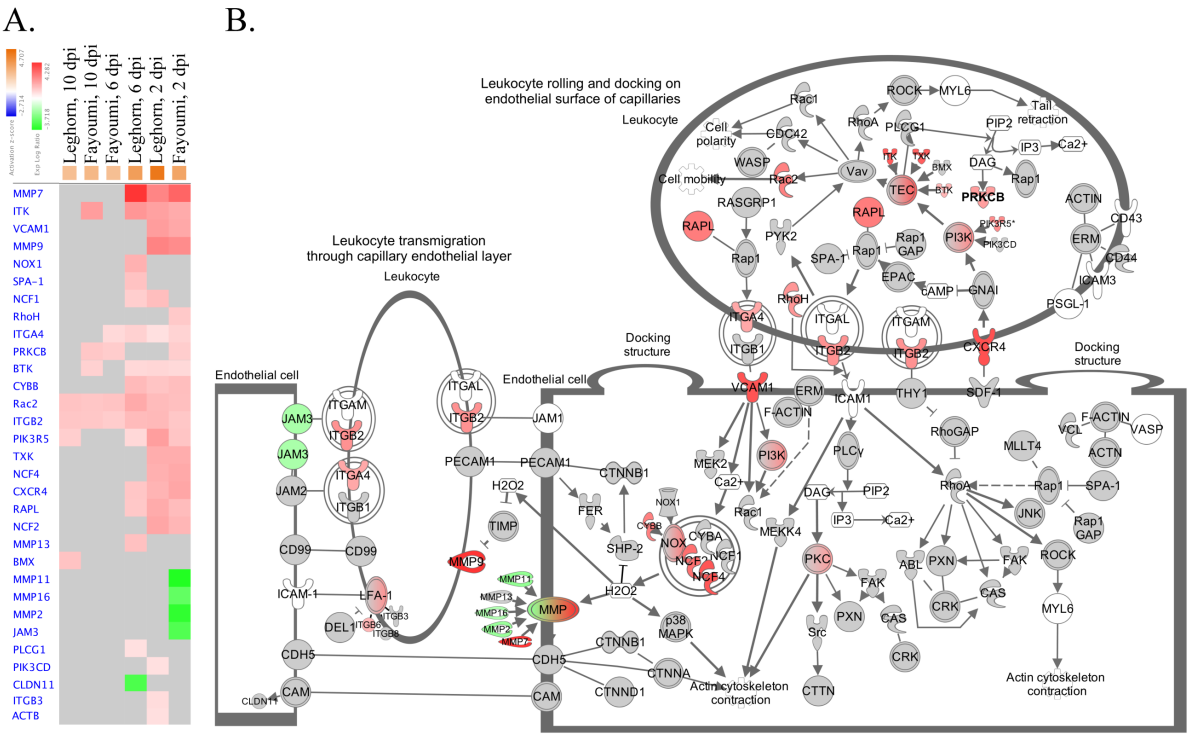

Figure 2-5: Leukocyte extravasation signaling pathway. All genes absolute LFC>1, FDR<0.05. A. Gene expression heat map of genes in the Leukocyte extravasation signaling pathway generated by contrasting the challenged vs. nonchallenged birds within each time and line. The contrasts, listed atop each column, were clustered by similarity based on genes (blue text) that were upregulated (red), downregulated (green), and had no change (gray). Genes that were not DE in any of the comparisons were removed from the graph. This pathway was activated in all contrasts (orange) as predicted by IPA®. B. The leukocyte extravasation signaling pathway in the Fayoumis at 2 dpi. Genes were upregulated (red), downregulated (green), or not DE (gray) in the challenged relative to the nonchallenged chickens. Genes in white were not found in the data set because they were removed during normalization, unrecognized by IPA®, or not found in chicken. The shapes of the genes represent their function. The genes within a parent node that were DE in any of the six contrasts are shown individually next to the parental node and are denoted with smaller molecules and labels.

Temporal changes influenced the pathways that were activated or inhibited in each line.

The top canonical pathways predicted in IPA® are shown in Figure 2-6 for each of the six major contrasts. The six major contrasts were clustered by similarity, which was determined by the resemblance in the activation or inhibition scores of all the pathways. The clustering grouped the major contrasts by dpi, with the Fayoumis at 6 dpi being the outlier (Figure 2-6). At 6 dpi there were only 119 DEG for the Fayoumis to populate the pathways, making it more difficult to obtain significance for activation or inhibition of a pathway. Several key pathways were significantly activated at all time points in both lines: iCOS-iCOSL signaling in T helper cells, NF-Kβ signaling, role of NFAT in regulation of the immune response, calcium-induced T lymphocyte apoptosis, phospholipase C signaling, CD28 signaling in T helper cells. These conserved pathways may be inherently crucial to the chicken's response to NDV in the trachea. The SAPK/JNK signaling pathway was only activated in the Leghorns and at all three time points. This pathway may be associated with the Leghorns' susceptibility to NDV (Figure 2-6).

Collagen downregulation at 2 dpi was only identified in the Fayoumis.

The network shown in Figure 2-7 shows the overwhelming downregulation of collagen, which was predicted by IPA® to be associated with the diseases and functions such as connective tissue disorders, dermatological diseases and conditions, organismal injury and abnormalities. This network had 25 focus modules and had the second highest score of the networks generated from this contrast. Of the 30 genes in this network, only 8 were also DE in the Leghorn (Figure 2-7). It does not appear that the downregulation of collagen was limited to a specific collagen type.

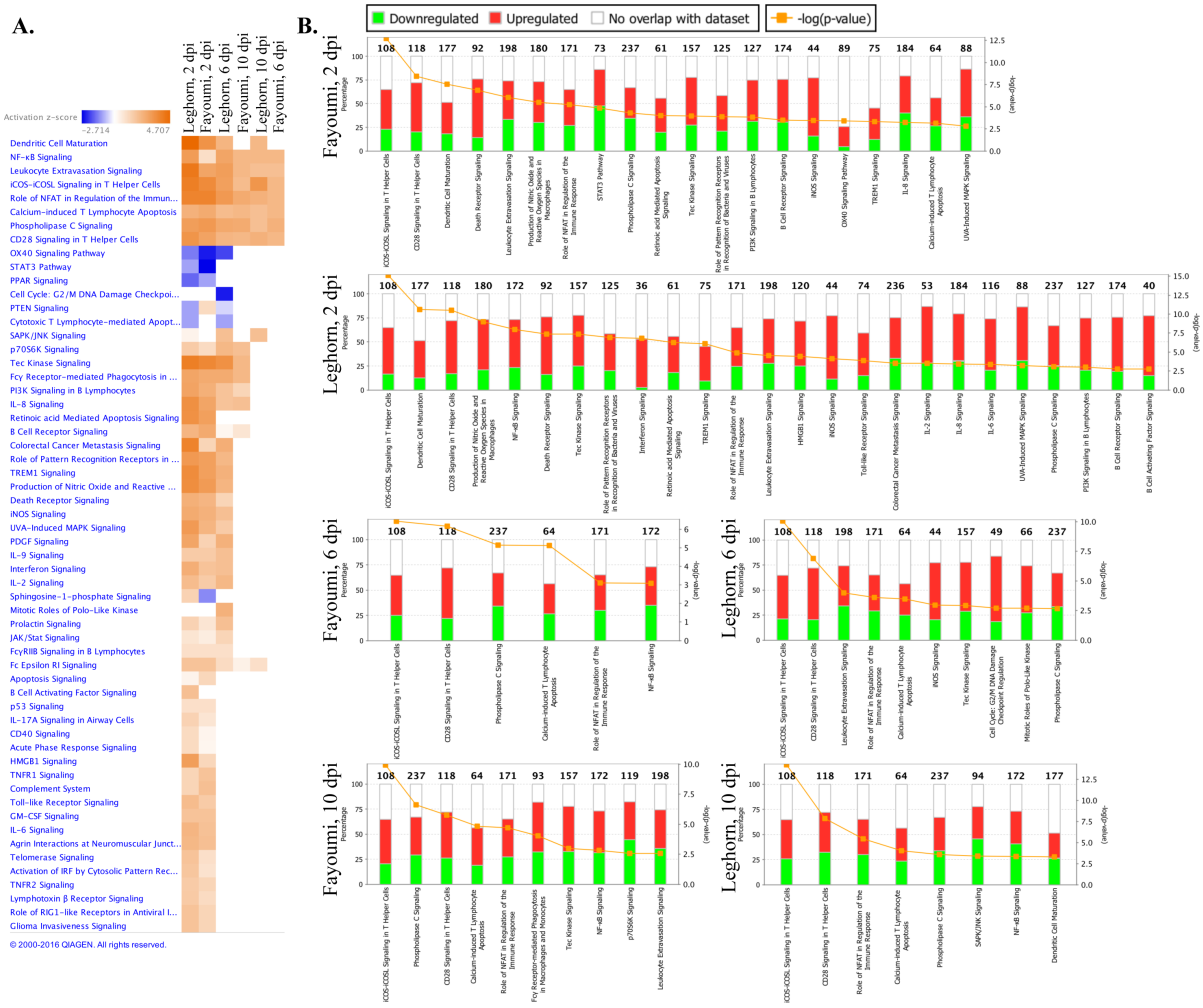

Figure 2-6: Pathways predicted to be activated or inhibited in response to NDV in the six major contrasts. All genes absolute LFC>1, FDR<0.05. A. Heat map, generated by IPA®, shows pathways that are either activated (orange), inhibited (blue), or unchanged (white) when the challenged and nonchallenged birds are contrasted within each line at the three separate time points. The pathways are sorted by hierarchical clustering and the contrasts (columns) are clustered by similarity. Pathways are filtered by -log(p-value>2.5), Z-score>1. B. The top pathways (-log(p-value)>2.5, Z-score>2) for each contrast are shown. The total number of genes in each pathway is found at the top of each bar, which is divided into genes that are downregulated (green), upregulated (red), and not in the gene set (white) by percentage of the total number of genes in each pathway. The orange line represents the –log(p-value) of each pathway.

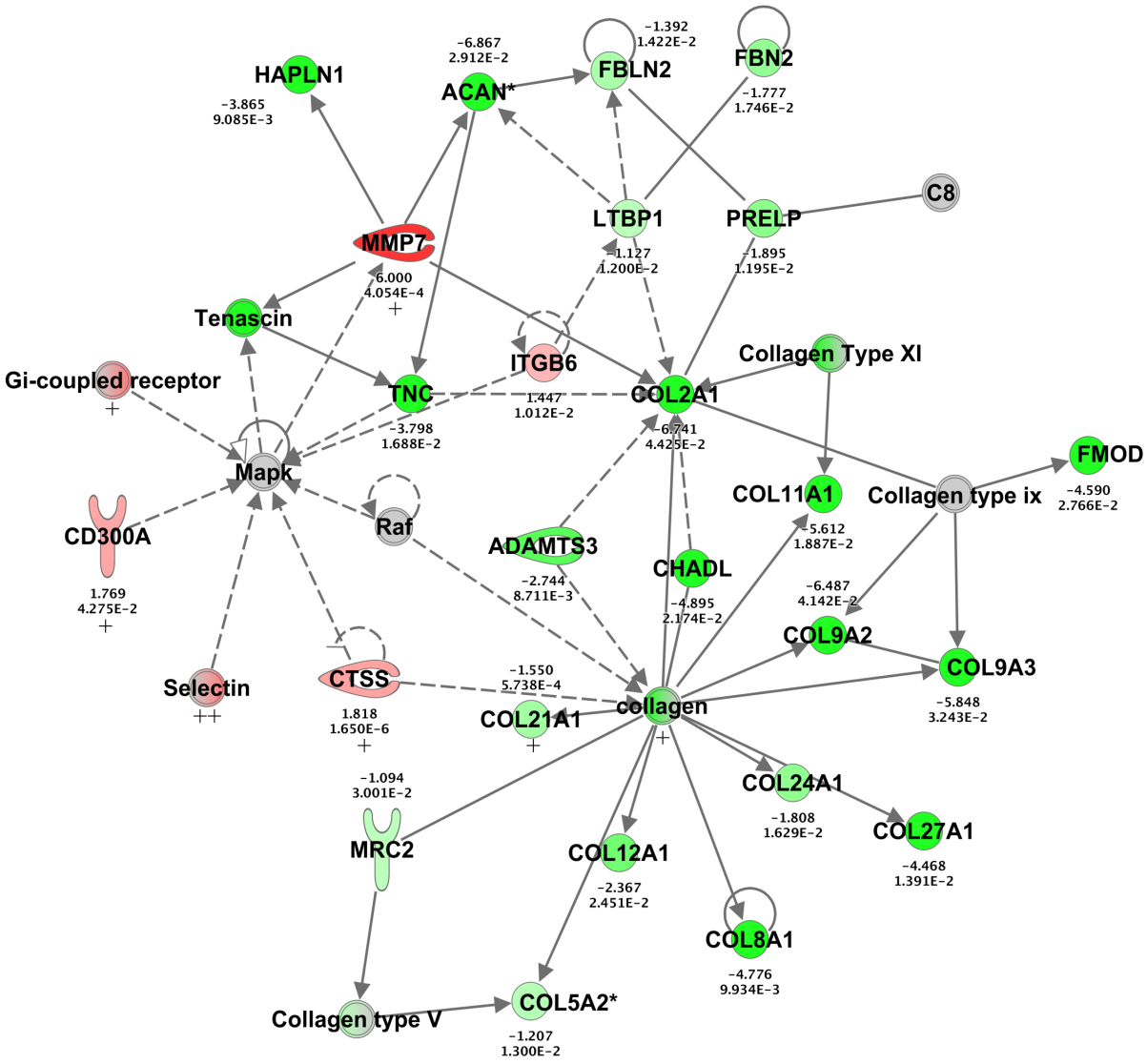

Figure 2-7: Collagen related network generated with from the Fayoumi challenged vs. nonchallenged chickens at 2 dpi. All genes absolute LFC>1, FDR<0.05. This network shows direct (solid lines) and indirect (dashed lines) relationships between genes that were either upregulated (red), downregulated (green), or no change (gray) between the challenged and nonchallenged Fayoumis at 2 dpi. The LFC (top) and FDR (bottom) are shown for each gene. Of the 30 genes, 8 genes denoted with a "+" were also DE in the Leghorns at this time.

Direct comparison of the Fayoumi and Leghorn at 2 dpi gave more insight into pathways and genes associated with resistance.

The Fayoumis and Leghorns had high similarity in enriched cell populations at 2 dpi (Figure 2-4), allowing for direct comparison between the two lines. The number of DEG between the Fayoumis and Leghorns was higher in the challenged birds than the nonchallenged (Figure 2-8). Myosin and troponin related genes, as well as MHC class I were the most upregulated genes in the Fayoumis relative to the Leghorns at this time. The eukaryotic translation initiation factor 2 (EIF2) signaling pathway had the highest positive Z-score (Figure 2-8), suggesting strong activation of this pathway in the Fayoumi relative to the Leghorn.

Examining the genes that were impacted by challenge and line offered potential candidate genes of interest.

A contrast was written to compare the interaction between challenge and line at each time point. At each time point, only DEG from the challenged vs. nonchallenged contrast within each line were examined for the interaction (line*challenge). The majority of the genes with a False Discovery Rate (FDR)<0.05 for the interaction were from 6 dpi, and nearly half of all genes are uncharacterized proteins (Table 2-2). Further investigation into the function of these uncharacterized proteins would be useful to provide more insight into the Fayoumi and Leghorn's resistant and susceptible phenotypes.

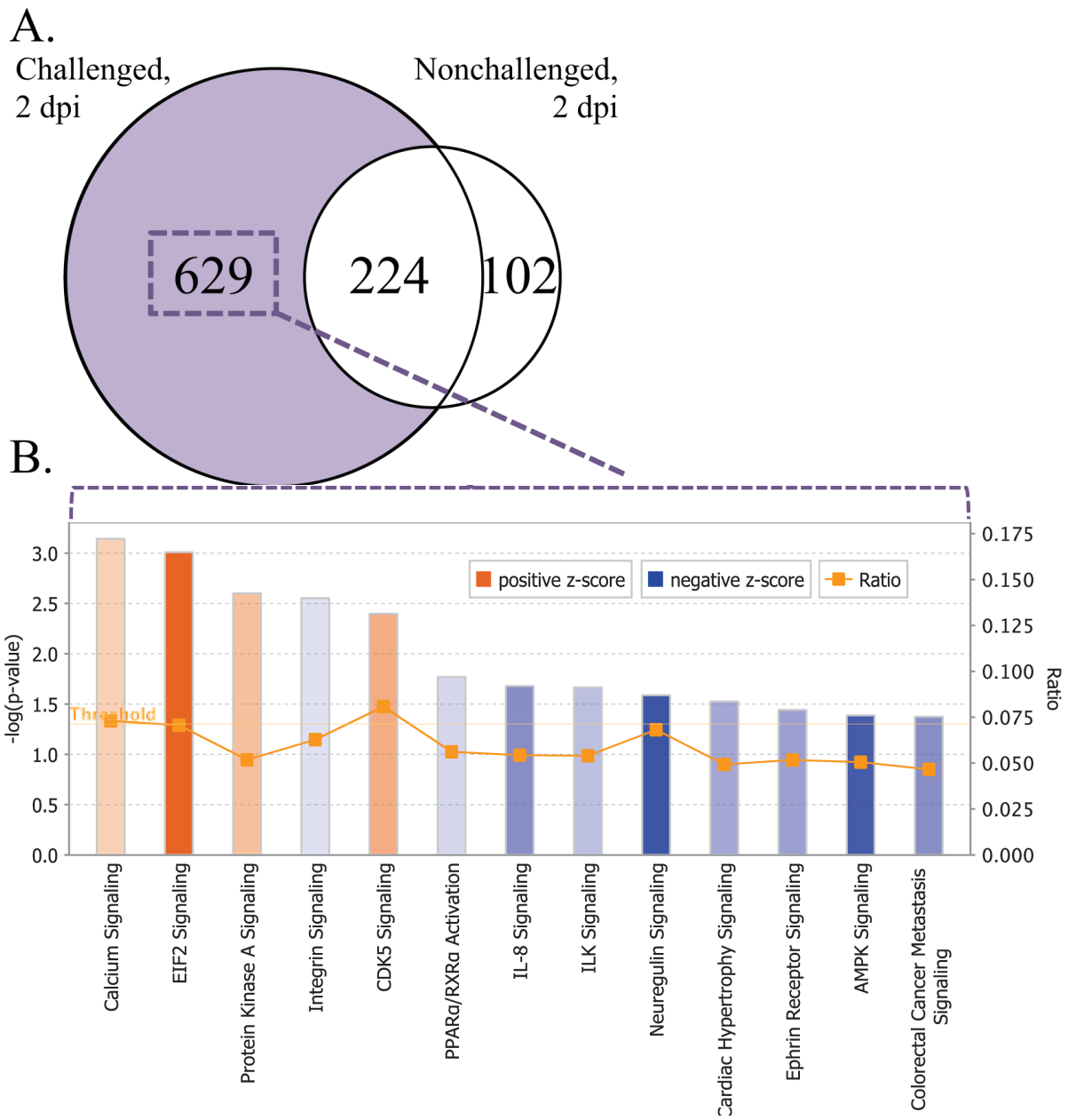

Figure 2-8: Contrasting the Fayoumi and Leghorn at 2 dpi. A. Venn diagram showing differentially expressed genes (FDR<0.05) between the Fayoumi and Leghorns in the challenged group (left) and nonchallenged group (right). The 629 genes (purple) that were uniquely identified as DE in the challenged group were used for pathway analysis. B. Top Canonical Pathways of the genes differentially expressed (FDR<0.05) between lines at 2 dpi in the challenged birds. Canonical pathways (Z-score>0.05, -log(p-value)>1.3) in orange are more likely to be activated in the Fayoumis and in blue are more likely to be activated in the Leghorns. The height of each bar corresponds to the –log(p-value) associated with each pathway and the lower the transparency of the bars' fill, the higher absolute value of the Z-score. The ratio (yellow line) represents the proportion of genes within the pathway that are DE.

Table 2-2: Genes whose expression level was significantly affected by challenge and line

Ensembl Transcript ID	Gene Name[a]	LFC[b]	FDR[c]	DPI[d]	Description[a]
ENSGALT00000015621	DMBT1	3.09	0.00574	2	Uncharacterized protein
ENSGALT00000034031		-5.82	0.00004	6	Uncharacterized protein
ENSGALT00000009555		-4.73	0.00034	6	Uncharacterized protein
ENSGALT00000018840	IGJ	-4.63	0.00034	6	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides precursor
ENSGALT00000034032		-5.11	0.00046	6	Uncharacterized protein
ENSGALT00000009519		-4.69	0.00052	6	Ig lambda chain V-1 region
ENSGALT00000009564		-4.81	0.00052	6	Uncharacterized protein
ENSGALT00000002609	GABRA1	4.48	0.00064	6	Gamma-aminobutyric acid receptor subunit alpha-1
ENSGALT00000008309	STX3	-2.86	0.00148	6	syntaxin
ENSGALT00000011371		-3.08	0.00351	6	Uncharacterized protein
ENSGALT00000003541		-4.45	0.00416	6	Uncharacterized protein
ENSGALT00000004568	ENPP3	-3.98	0.00705	6	ectonucleotide pyrophosphatase/phosphodiesterase 3
ENSGALT00000043739		4.41	0.01388	6	Uncharacterized protein
ENSGALT00000025174	DOK7	2.11	0.01447	6	docking protein 7
ENSGALT00000002058	TEKT3	3.89	0.01473	6	tektin 3
ENSGALT00000022671		-1.45	0.01473	6	Uncharacterized protein
ENSGALT00000034018		-4.12	0.01984	6	Uncharacterized protein
ENSGALT00000040746	TNFRSF13B	-4.29	0.03047	6	tumor necrosis factor receptor superfamily member 13B
ENSGALT00000038130	HEPACAM2	1.97	0.03127	6	HEPACAM family member 2
ENSGALT00000037100	CTNND2	1.76	0.03708	6	catenin delta 2
ENSGALT00000043820	MZB1	-3.68	0.03827	6	marginal zone B and B1 cell-specific protein
ENSGALT00000006673		-1.88	0.04387	6	Uncharacterized protein
ENSGALT00000036627		1.71	0.04387	6	cHz-cadherin precursor
ENSGALT00000012349	MEGF11	1.51	0.0444	6	multiple EGF-like-domains 11
ENSGALT00000027128	SULT1C	-1.39	0.04901	6	sulfotransferase 1C1
ENSGALT00000031381		1.66	0.04901	6	Uncharacterized protein
ENSGALT00000009564		-4.86	0.00638	10	Uncharacterized protein
ENSGALT00000043908		3.33	0.00638	10	Uncharacterized protein
ENSGALT00000030326	NYX	4.54	0.03834	10	nyctalopin
ENSGALT00000038746	HDC	2.67	0.03834	10	histidine decarboxylase

[a]Ensembl BioMart was used to obtain Gene Name and Description from the transcript ID.
[b]Log Fold Change (LFC), [c]False Discovery Rate (FDR), [d]Days post infection (DPI)

The RNA-seq results were validated with an independent test.

Fluidigm® Biomark HD was used as a method of high-throughput quantitative-PCR to serve as validation for the RNA-Seq results. The expression of 44 genes (Table 2-3) was analyzed. Figure 2-9 shows a correlation of 0.91 between the two methods across all six major contrasts. The high correlation serves as a validation of the RNA-seq technology applied in this study.

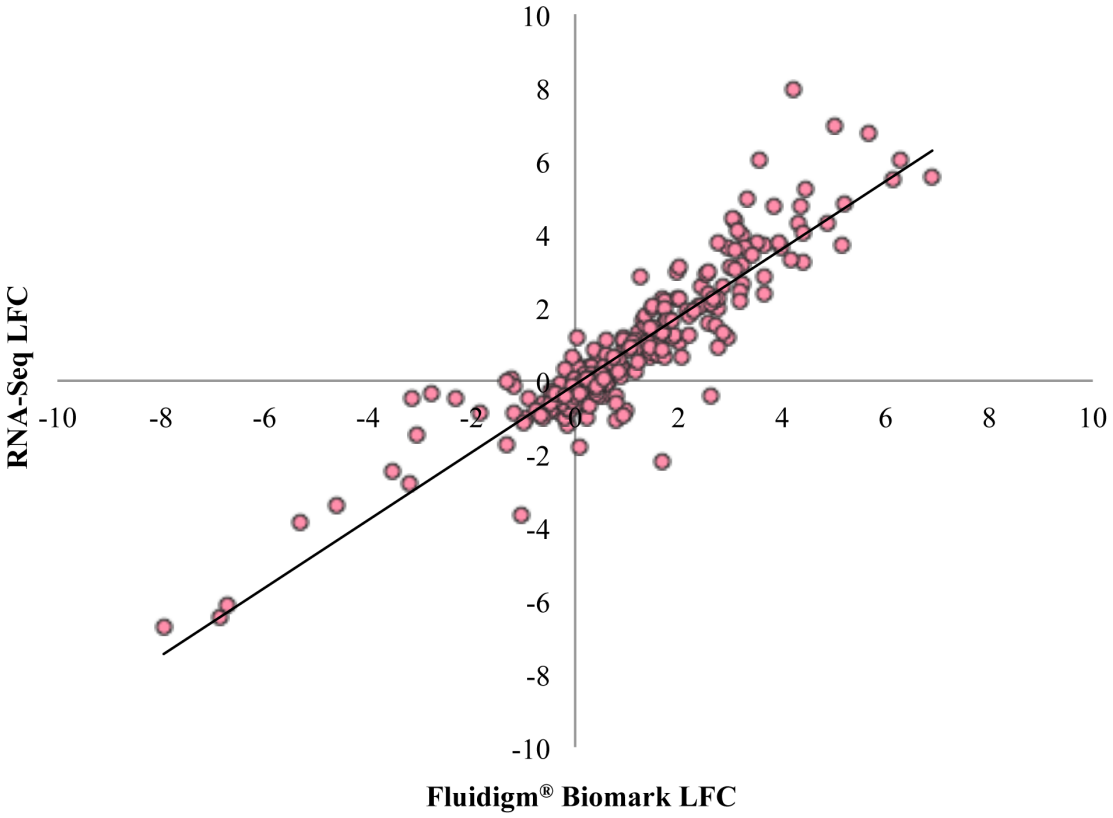

Figure 2-9: RNA-Seq results validated using Fluidigm® Biomark System. This scatter plot shows the agreement between the $\log_2$ fold changes (LFC) measured by RNA-Seq and the Fluidigm® Biomark system. The LFC were generated from the six major contrasts, the challenged and the nonchallenged at each time and in each line, for 43 genes in total. Each dot represents the LFC of a gene in a particular contrast; there are 258 points total. The pairwise correlation between the two methods was 0.91. H6PD was the housekeeping gene used for normalization of the Fluidigm® Biomark data. See Table 2-3 for primer information.

Table 2-3: Primers used for validation of RNA-seq data.

Gene Name	Forward Primer	Reverse Primer
ACKR2	GACATCCAGCTCTCAGAGACA	CACGTGTTGGTGATGCTCAA
AJAP1	GCCTGGAGATTACAAAGCAACC	AGACACAAAGGCCACAGGAA
APOA1	CCCTCGCTGTGCTCTTCC	GCCTTCACCGTCTCCAGGTA
CASP7[a]	ATGACCGAAGCTGTGAGGATA	GGCAAAACAAGCAGCATCAC
CASP9[a]	TTTCAGGTCCCTGTGCTTCC	TTCCGCAGCTCCACATCAA
CCR8	TCTGTGAGGCTTTCGGCAAA	TCCGTGTTGCGTCTGTTGAA
CD4	AGTGGAACCTGGATGTGTCA	TTTCCAAGCGTTCCTTCTCAAA
CD40[a]	AGCCTGGTGATGCTGTGAA	CTCACAGGGTGTGCAGACA
CMPK2	AGGCTGAACTGGAAGCTAACA	CTTGGCACGCAGGATTCAC
COL2A1_s5	GGTCCTTCCGGCTTCCA	CAGGAACACCCTGGTCTCC
COL5A2	TGGGAACCACCTGACACAAA	GAGCCAAACGTCAGCTTCAA
COL9A2	CCAAGGCTTGCCAGGAGTCA	CCTTTGGGACCGGTCTTTCC
CREB3L1	ATGCTGAAACCAGCGAGGTA	AACAGCTGCCTGCTCACTTA
CRISP3	AGGCGAGACTTCCAATCTTCC	GACAGCACAGCTGCAAGAC
H6PD[a]	ATGTACCGGGTGGACCACTA	AACTGACGGTTCTGATCTCGAAA
HCK	ACTGGAAACAGAGCTCAGAGAC	TCTGCAGGAAGCAGAGAAACA
IL18[a]	CGTGGCAGCTTTTGAAGATGTA	CTGAATGCAACAGGCATCCC
IL1B[a]	TGCTTCGTGCTGGAGTCAC	GGCATCTGCCCAGTTCCA
IL6[a]	AACGTCGAGTCTCTGTGCTA	AGTCTGGGATGACCACTTCA
IL8[a]	CCCCACTGCAAGAATGTTGAAA	GTGCCTTTACGATCAGCTGTAC
ITK	TGGAACAAGTGCCAGACCAA	AAGTCGCTATGGCTTTGGCTA
LAG3	CAGAACCAGAGCAGCAGAAAC	CAACAGTGACAGCACAGCAA
LITAF[a]	ATCGTGACACGTCTCTGCTA	AGCATCAACGCAAAAGGGAA
MAPK8IP3[a]	GCCAAAGCCAAAATGGAAACC	GTCGAGCAACAATCGCTTCA
MHCI-likeY	GCCGGAACGCTACAACAAA	TCCAGGATGTCACAGCCAAA
MMP7	CGGGACAGGCAGACATCA	TGTGCCACCTCTTCCATCAA
MOV10	CAAGAAGCTGTTGAAGCCCTAC	AGGAAAGTTGGCCGATACCA
Mx	CTGTTGCGATGCTGAACAAA	TTAGCAAAACGCTTGTTAGCC
MYO7A	TGAGGCAGGAATCCTTAGGAAC	ACTGCCACCAGTATTGAACCA
NLRC5[a]	TTTGCTGCTGCGCTTTCA	TGTGATGCTTCCACCTGTCA
PRKCB	ATGTGGTACAGATGCCAACCA	AGCCCCAAACAGAACGGTAA
RHPN2	TGACCGCACATACCGTAACA	AAGAGCTGTCCTGAACCAACA
RPL4[a]	TTCTGCCTTGGCAGCATCA	AGGAAGTTCTGGGATCTCCTCA
SDHA[a]	CGTGATCTGGCTCATCTAAAGAC	TTGCAGTTCAAGGGTCTCCA
SERPINB2[a]	AAATCACCGTGCCTGTTTCC	GTGCTTTAAACCCAGCATGGATA
SLC16A6	TCACAGCAGTGGCATCTACA	CAGCCAATCTGCTCCTTCAAA
SPON2	TCACACCCAAGGTGGGAAAA	ACTCTGTGCTCCCTGCAAA
TGFB3[a]	GGGCCCTGGATACCAACTAC	GGTCCTGTCGGAAGTCAATGTA
TLR4[a]	CCTGCTGGCAGGATGCA	TGTTCTGTCCTGTGCATCTGAA
TRAF6[a]	TGCCCCAGTACCATGCTTTTA	GTGTCGTGCCAGTTCATTCC
TRAT1	CGTAGCACAGCACAGAAACA	ATTGTCCCCTGCTTCACTCA
WNT4[a]	AGCAAAGGGGCATCTTCCAA	TCCACCCGCATGTTGTTCA
Worthington	TTCACAGGCACCTACATCACA	GGTGTTTTGTGTCCCATGCA
ZNFX1	TGCTGAAGTCTGCTGCTGTA	AACATTGGCCGTTCACTGAC

[a]Primers were used/analyzed previously (62).

Discussion

Examining changes in the transcriptome of the tracheal epithelia between challenged and nonchallenged birds offered valuable insight into host response to NDV. This study predicted the enrichment of immune cells in the trachea in challenged birds of both lines at all times. One pathway that is instrumental in leukocyte migration is leukocyte extravasation signaling. Based on the expression of genes within this pathway, IPA® predicted the activation of actin cytoskeleton contraction and cell mobility. A previous study examining gene expression levels in the lung after challenge with AIV in the same experimental lines showed an upregulation of actin filament-based movement in the Fayoumis (29). Actin cytoskeleton activation is crucial for cell migration as leukocytes use actin polymerization and actomyosin contraction for movement. Notably, paramyxoviruses like NDV also require actin to replicate efficiently (32, 33). If the migrating cells become infected with the virus, this may create a conundrum for the host. In one study, there was a downregulation of actin related genes after challenge with NDV *in vitro* (34). Due to challenge with Infectious Bronchitis Virus (IBV), proteins involved in cytoskeleton organization differed in expression in the trachea depending on IBV virulence (35). The host must find a way to balance the costs and benefits of cytoskeleton upregulation with defense against viral infection. The regulation of cytoskeleton and actin related genes may be time dependent and may be a critical target for future study.

Host-pathogen interactions at the site of infection can determine the severity of both the pathology and the overall infection. The use of two unique inbred lines that differ in their relative resistance offered a valuable tool of comparison. Fayoumis had significantly less detectable virus at 2 dpi (measured by RNA-seq) and 6 dpi (measured by qPCR), which may result in faster viral clearing, and overall supports their relatively resistant phenotype when

compared to the Leghorns. Decreased viral load corresponds to less viral shedding, and therefore, fewer viral particles in the environment and lower transmission potential (36). In a separate study with the same two genetic lines, although all birds succumbed to infection with velogenic NDV, the mean survival time was longer for the Fayoumis than the Leghorns at all four doses tested, and was significantly longer at the two higher doses (P. Miller and C. Afonso, SEPRL, Athens, GA, 2016).

As time post-infection increased, the numbers of DEG between challenged and nonchallenged birds decreased, suggesting that the chickens were recovering. The Fayoumis had much fewer DEG at 6 dpi compared to the Leghorn, which may be further evidence that the Fayoumis recovered more quickly. Although most pathways had similar predicted activation or inhibition for the two lines, the lines often differed in gene expression levels within the pathway (Figure 2-5). The current study identified several genes for which their expression levels are impacted significantly by both line and challenge (Table 2-2), some of which overlap with a previous study. In the lung, TNFRSF13B and IGJ were DE between the Fayoumi and Leghorn and Ig lambda chain V-1 region was DE in both lines after challenged with AIV (29). These genes may be of particular importance in the host response to both viruses, AIV and NDV.

The wide range of pathogens (parasites, bacteria, viruses) to which the Fayoumis have shown relative resistance suggests the mechanism(s) for their resistance may function early in the immune response (26, 27, 29). Downregulation of collagen at 2 dpi in the challenged Fayoumis could be partially responsible for their resistant phenotype. One study found lower mRNA expression levels of collagen in the trachea of resistant chickens (non-inbred Cornell) compared to susceptible (inbred White Leghorn) chickens after challenge

with IBV (37). The downregulation of collagen may impact apoptosis (38), immune cell migration(39-41), and T-cell activation (42-46); impacting these processes would likely impact the virus and its pathogenesis.

Directly comparing the challenged Fayoumis and Leghorns at 2 dpi also gave insightful results. Integrin signaling was more highly activated in the Leghorns relative to the Fayoumis (Figure 2-8). This may be directly related to the downregulation of collagen and its relationship with integrin (38). Laminin, another key extracellular matrix protein (39), was also upregulated in the Leghorns relative to the Fayoumis. The impact of collagen was shown in two separate contrasts. The downregulation of collagen between challenged and nonchallenged Fayoumis, and the activation of collagen related pathways in the Leghorns relative to the Fayoumis gives more confidence in the importance of collagen at 2 dpi.

The activation of the PKR/EIF2 signaling cascade is known to inhibit NDV replication (47), likely due to the actions of the antiviral protein, PKR. This pathway, involved in mRNA translation, was more upregulated in the Fayoumis than the Leghorns (Figure 2-8). This finding was in agreement with the Fayoumi's resistant phenotype, and may be related to the lower viral load seen at 6 dpi. Phosphorylation of EIF2-a results in apoptosis due to a global termination of transcription (48). Apoptosis related pathways were activated including T lymphocyte apoptosis, p53 signaling, Retinoic acid mediated apoptosis signaling, and apoptosis signaling (Figure 2-6). The EIF2 signaling pathway was also identified as differentially methylated between chickens that were either vaccinated or not for Infectious Laryngotracheitis (48). It is clear that this pathway is critical in host defense to multiple pathogens.

In a previous study, chicken stem cells underwent rounds of selection to a high-titered La Sota strain; these selected cells' expression levels lead to increased apoptosis and decreased neuroactive ligand-receptor interaction when compared to the unselected cells (49). The Fayoumis showed higher activation of apoptosis signaling at 2 dpi (Figure 2-6) and when the lines were directly compared the Leghorns showed more activation of the neuregulin signaling pathway at 2 dpi (Figure 2-8). The overlap in these pathways suggests that increased levels of apoptosis and decreased neurological related pathways may be important mechanisms of host resistance to La Sota.

The trachea transcriptome exposed potential key mechanisms for host defense against NDV. As expected, there appeared to be a large increase in immune cell infiltration in the trachea in both lines at all times examined. Although there were shared mechanisms seen in both the Fayoumi and Leghorn, the lines showed unique responses as well. This study has demonstrated the Fayoumi's classification as relatively resistant to NDV compared to the Leghorns, as determined by viral load and viral sequence counts, and suggested by antibody titer and DEG numbers. This study identified possible mechanisms for the Fayoumi's resistance, including the downregulation of collagen and the increased activation of EIF2 signaling, which can be used to improve vaccine development and highlight genes for which beneficial genetic variance can be used to inform breeding decisions. Cytokine expression levels are time, tissue, and strain dependent (11-13). It is critical that future breeding decisions be made from data based on multiple tissues, host genetic backgrounds, NDV strains, and time points. The current study is a first step in understanding and identifying possible resistance mechanisms to NDV. Future studies to examine the gene expression of

Fayoumis and Leghorns challenged with velogenic NDV are necessary to better determine the applicability of the results presented.

Materials and Methods

Ethics Statement and Animals Used

Methods were approved by Iowa Sate University IACUC log #1-13-7490-G. Fayoumi (M 15.2) and Leghorn (GHs 6) chicken lines from Iowa State University Poultry Farm (Ames, IA 50011) were used in this study. The Fayoumis and Leghorns each have an inbreeding coefficient >99.9% as determined by microsatellites (50) and 99.95% by resequencing (51). The Fayoumis originated from the Fayoum region of Egypt, whereas the Leghorn line is representative of the U.S. egg laying industry of the 1950's. The chickens were given *ad libitum* access to water and feed throughout the study. Supplemental heat was provided to the birds starting at day of hatch at 35°C, and stepping down 2-3°C every few days until reaching 24°C at day 29 of age, and remaining at 24° C for the remainder of the study. Light cycle began at 23L: 1D at day of hatch, and the hours of light were gradually reduced until reaching 13.5 hours of light at 24 days at age, and remaining at that amount for the remainder of the study.

Virus

A live attenuated Newcastle disease vaccine, B1 type La Sota lentogenic strain was propagated once in 10-day-old embryonated specific pathogen free (SPF) chicken eggs inoculated via the allantoic sac (52). The allantoic fluid was harvested after two days, tested for virus presence by Hemagglutinating activity (HA) and centrifuged at 8,000 RPM for 30 minutes to clear debris. Virus titration was performed in SPF embryonated eggs as previously described (53). The final titer of the undiluted virus suspension was $10^8 EID_{50\%\ per}$ ml. The virus suspension was aliquoted and stored at -80°C until its use.

Experimental Design

At hatch, straight run chickens of both lines were randomly assigned to one of four rooms in a biosafety level II facility. At 21 days of age, half of the chickens (two rooms, n=49) were inoculated with 200 µL of $10^7 EID_{50\%}$ La Sota NDV, 50 µL inoculated into each of the eyes and nostrils. The nonchallenged group (2 rooms, n= 40) was given 200 µL phosphate buffered saline (PBS) via the same route, as a mock infection. Chickens that received the La Sota virus are referred to throughout the manuscript as "challenged" and those that received the mock infection, "nonchallenged." To collect lachrymal fluid, pre-challenge (n=89) and 2 (n=89) and 6 (n=62) dpi, fine, crystalline sodium chloride was placed on the eye of each chick to collect lachrymal fluid with a pipette. To quantify antibody levels, venous blood samples were collected pre-challenge and at 10 dpi. At 2, 6, and 10 dpi, 24, 30, and 35 chicks, respectively were euthanized with sodium pentobarbital solution for tissue harvest. The entire trachea was harvested from all of the challenged and nonchallenged birds sacrificed at each dpi and placed into RNA*later*® solution (ThermoFisher Scientific, Waltham, Massachusettes) for short-term storage. Within a week, the sheet of epithelial cells from each trachea was carefully removed by peeling from the interior surface of the trachea with forceps. This epithelial cell lining was placed into a -80°C freezer for long-term storage, and the remaining tissue was discarded.

NDV Antibody

Blood samples collected pre-challenge and at 10 dpi were centrifuged at 15 RPM for 5 minutes to collect the serum supernatant. From the serum NDV antibody levels were measured using the IDEXX NDV ELISA for chickens (IDEXX Laboratories, Inc., Westbrook, Maine). The Sample to Positive (S/P) ratio was calculated by subtracting the negative control from the average absorption value for each sample and dividing by positive

control value minus the negative control value. The positive and negative controls were supplied by the IDEXX kit. The S/P ratio was calculated from the average absorbance values of each sample, which was run in duplicate. A standard least squares, effect leverage analysis in JMP (JMP Group Inc., San Francisco, California) including the main effects of line, treatment, and line*treatment was used; a Student's T-test generated the connecting letters report. Both tests are parametric.

Viral Load

The viral RNA was isolated from chicken lachrymal fluid collected pre-challenge and 2 and 6 dpi using the MagMAX™-96 viral RNA Isolation Kit (Life Technologies, Carlsbad, California) and was quantified using the LSI VetMAX™ Newcastle Disease Virus Real-Time PCR Kit (Life Technologies, Carlsbad, California) on the MJ Research Opticon2 qPCR machine (Bio-Rad, Hercules, California). The primers were directed against the Matrix (M) gene and dilutions of inoculum virus were used as standards. The log copy number per 1 µL of isolated viral RNA was calculated for the mean of each sample, which was run in duplicate. Using JMP statistical software (JMP Group Inc., San Francisco, California) a standard least squares, effect leverage test with main effects including line, dpi, and line*dpi, was performed to determine significance, and a Student's T-test was utilized to generate a connecting letters report. The log copy number of the NDV genome does not show the number of infectious particles.

RNA-isolation and cDNA library construction

RNA was isolated from the tracheal epithelial cells using the RNAqueous® kit (ThermoFisher Scientific, Waltham, Massachusettes) and samples were then DNase treated using the DNA-*free*™ kit (ThermoFisher Scientific, Waltham, Massachusettes). The quality of all samples was validated (RNA quality number>8.0) using the Fragment Analyzer™

(Advanced Analytical Technologies, Ankeny, Iowa). A 500 ng input for each sample was utilized to construct the cDNA library using TruSeq® RNA Sample Preparation v2 Guide (Illumina, San Diego, California) using the high throughput protocol. Following cDNA library validation using the Fragment Analyzer™ (Advanced Analytical Technologies, Ankeny, Iowa), samples were sequenced on the HiSeq2500 platform (Illumina, San Diego, California) for 100 base pair, single-end, reads (DNA Facility, Iowa State University, Ames, Iowa).

RNA-seq design

This study included 3 main factors: treatment (challenged, nonchallenged), line (Leghorn, Fayoumi), and time (2, 6, 10 dpi), resulting in 12 treatment groups. Each treatment group was represented by 4 biological replicates, except for the Leghorns at 2 dpi, for which there were 3 nonchallenged and 5 challenged chickens included in the analysis. Equal numbers of males and females were used for each treatment group. The 12 treatments were balanced across four lanes of the flow cell and randomly assigned an index.

Data Analyses

The Discovery Environment of iPlant Collaborative™ (54) was utilized for data processing. First, FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was utilized for an initial quality check and assessment of all sequence data. The Illumina TruSeq adapter was recognized as an overrepresented sequence in some samples. For consistency, the adapter sequence and the individual multiplexing index was removed from all samples using the FASTX Clipper. In addition, FASTX removed any sequence less than 30 base pairs in length and filtered each read such that 80% of all base pairs within a read had a Phred quality score of 30 or higher. The remaining high quality reads were used as input for TopHat2 (55) for alignment to the Galgal4 reference genome (GCA_000002315.2). The

Galgal4 (GCA_000002315.2) and GTF file were downloaded from Ensembl. In TopHat2 (55), default settings were used, except the minimum isoform fraction was lowered to 0.10 (0.15 default) to identify more alternatively spliced transcripts. HTSeq (56) was used to count reads aligned to each transcript (mode: intersection-nonempty). Statistical analysis of the count data was performed using the generalized linear model option in edgeR (57). The main effects of line, treatment, and time were included in the model. The contrasts comparing the challenged and nonchallenged birds within each line, at each time will be referred to as the six major contrasts (Fayoumi,2dpi, Challenged vs. Nonchallenged; Leghorn,2dpi, Challenged vs. Nonchallenged; Fayoumi,6dpi, Challenged vs. Nonchallenged; Leghorn,6dpi, Challenged vs. Nonchallenged; Fayoumi,10dpi, Challenged vs. Nonchallenged; Leghorn,10dpi, Challenged vs. Nonchallenged).

The differentially expressed genes (DEG) (FDR<0.05) identified in either chicken line when comparing challenged to nonchallenged included 1,688, 965, and 180 DEG at 2, 6, and 10 dpi, respectively (Figure 2-3). For these genes, contrasts were written to determine the significance of the interaction between line and challenge at each time. The p-values generated from the interaction contrast were subjected to a Benjamini & Hochberg multiple testing correction (58).

NDV sequence discovery

NDV La Sota (JF950510.1) sequence was downloaded from NCBI and used to make a GFF file. Using the Burrows-Wheeler Alignment (BWA) (59) under default settings the unmapped reads were aligned to the NDV genome (JF950510.1). HTSeq (56) was used to count the number of reads that aligned to each viral gene. The cpm was calculated by taking the number of reads that aligned to each viral gene, dividing by total number of unmapped reads, and multiplying by one million. There was no statistical difference between the

number of unmapped reads in the challenged Leghorns and Fayoumis at 2 dpi (p=0.4). The data was imported into JMP statistical software (JMP Group Inc., San Francisco, California) and analyzed with a least squares analysis and Student's T-test to generate the connecting letters report.

Gene expression analysis

The trachea is a heterogeneous tissue composed of multiple cell populations that likely change under different treatment conditions. To determine cell type enrichment, the transcript IDs of DEGs (FDR<0.05) for each of the six major contrasts were converted into their associated gene names using BioMart (http://uswest.ensembl.org/biomart/martview/). The six gene lists were then input into CTen (http://www.influenza-x.org/~jshoemaker/cten/) (60). The CTen database is based on human and mouse data, and has been used previously in chickens (61).

For pathway analysis, QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) was used. The ID's of transcripts unrecognized by IPA® were converted to a recognized ID using BioMart (http://uswest.ensembl.org/biomart/martview/) to ensure the maximum transcripts were included for pathway analysis. Genes used for pathway analysis had a FDR<0.05 and absolute Log_2 Fold Change (LFC) >1, as estimated by edgeR.

RNA-seq data accession number

Gene expression data were deposited at ArrayExpress, EMBL/EBI under accession number E-MTAB-5431.

Fluidigm® Biomark HD

To validate the RNA-Seq results, the Fluidigm® Biomark HD (Fluidigm®, South San Francisco, California) was used as a method of high-throughput q-PCR. The input RNA, 25

ng/μL, came from the same isolate used for cDNA library construction. The 48 RNA samples were converted into cDNA, pre-amplified at 12 cycles with all 48 primer pairs (Table 2-3), treated with exonuclease, diluted 10-fold, and analyzed on the 48.48 IFC for a total of 2,304 sample x gene tests. The genes were chosen to represent the range of log fold changes, not all genes were DEG. Ribosomal protein L4 (RPL4) and hexose-6-phosphate dehydrogenase (H6PD) were two housekeeping genes with high raw cycle threshold (CT) value correlation (r=0.9). Since its CT values were closer to the average CT values of all primers and samples, H6PD was used as the housekeeping gene for the analysis. Data were filtered by Fluidigm® quality assessment and melting curve consistency. If after these filtering steps primer pairs did not have more than 28 samples remaining, all measurements for that gene were excluded. A total of 9.5% of the data, including four genes (MZB1, 5_8S_rRNA, UbI, and GAL2), were excluded from the analysis. Table 2-3 shows the genes and primers analyzed in this study.

For all six major contrasts, the log fold change (LFC) was calculated by $-2^{-\Delta\Delta CT}$ for each gene. The correlation between the RNA-seq generated LFC and the Fluidigm® Biomark generated LFC was determined with a pairwise correlation across all genes and all six major contrasts, 258 pairwise comparisons total.

Author contributions

MSD: Collected samples, isolated RNA, constructed cDNA libraries, processed and analyzed RNA-seq data, wrote paper
RAG: Experimental design, prepared viral isolate for inoculation, reviewed and edited paper
DAB: Experimental design, reviewed and edited paper
TRK: Reviewed and edited paper
JCMD: Experimental design, advised on statistical analyses, reviewed and edited paper
HZ: Experimental design, reviewed and edited paper
SJL: Experimental design, reviewed and edited paper, oversaw analysis of data

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CHAPTER 3. RESISTANT AND SUSCEPTIBLE CHICKEN LINES SHOW DISTINCTIVE RESPONSES TO NEWCASTLE DISEASE VIRUS INFECTION IN THE LUNG TRANSCRIPTOME

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Abstract

Background:

Newcastle disease virus (NDV) is a threat to poultry production worldwide. A better understanding of mechanisms of resistance and susceptibility to this virus will improve measures for NDV prevention and control. Males and females from resistant Fayoumi and susceptible Leghorn lines were either challenged with a lentogenic strain of the virus or given a mock infection at three weeks of age. The lung transcriptomes generated by RNA-seq were studied using contrasts across the challenged and nonchallenged birds, the two lines, and three time points post-infection, and by using Weighted Gene Co-expression Network Analysis (WGNCA).

Results:

Genetic line and sex had a large impact on the lung transcriptome. When contrasting the challenged and nonchallenged birds, few differentially expressed genes (DEG) were identified within each line at 2, 6, and 10 days post infection (dpi), except for the more resistant Fayoumi line at 10 dpi, for which several pathways were activated and inhibited at

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this time. The interaction of challenge and line at 10 dpi significantly impacted 131 genes (False Discovery Rate (FDR) <0.05), one of which was *PPIB*. Many DEG were identified between the Fayoumi and Leghorns. The number of DEG between the two lines in the challenged birds decreased over time, but increased over time in the nonchallenged birds. The nonchallenged Fayoumis at 10 dpi showed enrichment of immune type cells when compared to 2 dpi, suggesting important immune related development at this age. These changes between 10 and 2 dpi were not identified in the challenged Fayoumis. The energy allocated to host defense may have interrupted normal lung development. WGCNA identified important modules and driver genes within those modules that were associated with traits of interest, several of which had no known associated function.

Conclusions:

The lines' unique response to NDV offers insights into the potential means of their resistance and susceptibility. The lung transcriptome shows a unique response to lentogenic NDV compared to a previous study on the trachea of the same birds. It is important to analyze multiple tissues in order to best understand the chicken's overall response to NDV challenge and improve strategies to combat this devastating disease.

Background

Chickens offer a relatively environmentally friendly and healthy source of protein in form of both meat and eggs [1, 2]. From backyard flocks to commercial settings, chicken production is scalable. In smaller flocks, chickens are often scavengers, easing management; however, this setting also increases risk of the chickens coming into contact with pathogens, as biosecurity practices are rare. In commercial settings, pathogens spread rapidly due to close quarters of animals. Disease is a continuous threat to food security and also to human health in the cases of zoonotic pathogens.

One alternative strategy to curb the devastating impacts of disease in chickens is to utilize host genetic variation to facilitate breeding for disease resistance. Disease resistance is often not absolute; here it is defined as the ability of an individual chicken to interfere with the pathogen life cycle [3]. Examples of phenotypes associated with disease resistance include lower pathogen load, higher antibody titer, or less morbidity and/or mortality. Mounting an immune response consumes large amounts of energy that the chicken cannot put towards production traits such as growth and egg laying [4]. Ideally, disease resistant chickens will expend less energy in the face of a disease challenge and have enough energy to continue to grow and lay eggs; this trait is called resilience [3].

Selection experiments on immune related traits have been successful, but often it is not known what genes and pathways are being selected upon. This may lead to improvement in resistance to one disease at the cost of increasing susceptibility to another [5].

Genetic solutions work best in combination with other disease control strategies, such as biosecurity, vaccination, and culling. When one or more of those strategies are not options, more emphasis should be placed on the others. In developing countries, where many flocks are scavenging chickens, vaccination to Newcastle disease (ND) is not feasible. Newcastle disease, caused by Newcastle disease virus (NDV), a negative sense, single stranded, RNA virus, results in a variety of symptoms and can result in mortality rates as high as 80% from highly virulent strains [6]. This virus is therefore a threat to food security. Fortunately, previous studies have shown that genetic differences result in different disease outcomes in response to NDV [7-10].

Two experimental chicken lines, Fayoumi and Leghorn, have been classified as relatively resistant and susceptible, respectively, to NDV [11] and to many other pathogens,

including avian influenza virus (AIV), Marek's disease virus, *Salmonella*, and *Eimeria* [12-15]. These inbred lines were used as a discovery platform to identify genes and pathways that may be associated with resistance to NDV via RNA-seq. Characterization of these two lines' response to NDV is necessary in order to identify possible explanations for their differing response. Previously, the trachea transcriptome from these two inbred lines was analyzed after challenge with lentogenic NDV [11]. To gain a more holistic interpretation, multiple tissues should be examined.

In this study, the lung was chosen for RNA-seq analysis due to its proximity to the site of infection (ocular/nasal), known immune importance [16], and because lentogenic NDV has been classified as a respiratory disease [17]. Lentogenic viruses replicate at the site of infection and require a trypsin-like protease to cleave the fusion protein, a viral protein required for entry into the host cell [18]. The host innate immune system rapidly produces cytokines, chemokines, and interferons in response to the virus, and triggers the adaptive immune response. Antibodies were detected as early as 4 days post infection in the saliva of Leghorns infected with lentogenic NDV [19]. Also, the lung is home to bronchus-associated lymphoid tissue (BALT), which plays a crucial role in antiviral immunity [20], and respiratory immune responses are induced in the lung [16]. Previous studies have examined the lung transcriptome in response to challenges with laryngotracheitis virus, AIV, and infectious bronchitis virus (IBV) [21-24]. Comparing across studies can help identify genes and pathways of importance to multiple viral pathogens in poultry.

Methods

This study utilized two inbred lines (inbreeding coefficient=99.95% [25]), Fayoumi (M 15.2) and Leghorn (GHs 6), from the Iowa State University Poultry Farm (Ames, IA).

Originating from Egypt, the Fayoumi has a history of harsh natural selection, while the Leghorn has a history of artificial selection for egg-laying traits. For the past 60 years, the only selection pressure placed on the two lines has been for survival and reproduction.

The methods used in the present study were approved by the Iowa State University IACUC (log number 1-13-7490-G) according to the appropriate animal guidelines [26]. At hatch, Fayoumis and Leghorns were placed into a biosafety level II facility and randomly assigned to one of two treatment groups. At three weeks of age the challenged birds (n=49) were inoculated via an ocular nasal route with 200 μL of $10^7 EID_{50}$ of La Sota NDV, 50 μL into each eye and nostril. The nonchallenged birds (n=40) were given 200 μL of phosphate buffered saline (PBS) via the same route, as a mock infection. The challenged and nonchallenged birds were kept in separate rooms. Four ABSL-2 rooms, two per treatment group, were used to house the birds. There was one floor pen in each room, with 20 to 24 birds per pen.

At 2, 6, and 10 days post infection (dpi), one-third of the birds were euthanized for tissue collection. The lung was removed, briefly diced, and placed in RNAlater (ThermoFisher Scientific, Waltham, MA) for short-term storage. Within a week, the samples were removed from the RNAlater solution and placed into a -80°C freezer.

Three main factors were included in this study: treatment (challenged, nonchallenged), line (Fayoumi, Leghorn), and time (2, 6, 10 dpi). The three time points were chosen to enable the observation of mechanisms of resistance during both the innate and adaptive immune response. In most cases, the number of males and females were balanced within each treatment group (Table 3-1).

Table 3-1: Sample sizes per treatment group used for RNA-seq analysis.

Treatment Group	Males	Females
Fayoumi, Nonchallenged, 2 dpi	2	2
Fayoumi, Challenged, 2 dpi	2	2
Fayoumi, Nonchallenged, 6 dpi	3	1
Fayoumi, Challenged, 6 dpi	2	2
Fayoumi, Nonchallenged, 10 dpi	2	2
Fayoumi, Challenged, 10 dpi	2	2
Leghorn, Nonchallenged, 2 dpi	2	1
Leghorn, Challenged, 2 dpi	2	3
Leghorn, Nonchallenged, 6 dpi	2	2
Leghorn, Challenged, 6 dpi	2	2
Leghorn, Nonchallenged, 10 dpi	2	2
Leghorn, Challenged, 10 dpi	2	2

Total RNA was isolated from the lung using an RNAqueous kit (Thermo Fisher Scientific, Waltham, MA). The isolated RNA was then DNase treated using a DNA-free kit (Thermo Fisher Scientific, Waltham, MA). All samples had an RNA quality number greater than 8.0, as measured using the Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies, Inc., Ankeny, IA). A 500-ng input was utilized to construct a cDNA library for each sample, using the high-throughput protocol in the TruSeq RNA sample preparation guide (v2; Illumina, San Diego, CA). The cDNA libraries were validated using the Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies, Inc., Ankeny, IA), and then sequenced on the HiSeq2500 platform (Illumina, San Diego, CA) for 100-bp, single-end reads (DNA Facility, Iowa State University, Ames, IA). The 12 treatment groups were balanced across four lanes of the flow cell and randomly assigned an

index. Sequence data can be accessed at the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5859.

Analysis of the RNA-seq data was performed in the Discovery Environment of iPlant Collaborative [27], now known as Cyverse. The Illumina TruSeq adapter sequence and the individual multiplexing indexes were removed from each sample using the FASTX Clipper program. Sequences less than 30 bp in length, or that did not have a Phred score greater than or equal to 30 in 80% of all bp were filtered out using FASTX. Remaining high quality reads were input into TopHat2 [28] (default parameters; isoform fraction=0.10) and aligned to the Gallus_gallus-5.0 (Gal5; GCA_000002315.3) reference genome that was downloaded from the Ensembl genome browser. Read counts for each transcript were generated from HTSeq [29] (mode: intersection-nonempty).

For data visualization, pcaExplorer [30] was used to generate principal component analysis (PCA) plots based on DESeq2 [31] normalized counts, while accounting for line, treatment, dpi, and sex using the dds function and the variance stabilizing transformation (vst). The 500 most variant transcripts were used to calculate the variance associated with each principal component.

The count data were statistically analyzed using the generalized linear model option in edgeR [32], accounting for line, treatment, dpi, and sex, to determine the number of differentially expressed genes (DEG) among the 12 treatment groups. A false discovery rate (FDR) less than 0.05 was used to declare DEG. Contrasts were written to compare the challenged and nonchallenged birds, the Fayoumis and Leghorns, and the different time points. A contrast was also written to determine which transcripts were significantly impacted by a challenge*line interaction at each time and these were input into STRING

(version 10.0) [33] for network analysis. In STRING, a medium confidence level (0.400) was utilized and disconnected nodes were removed.

Other programs utilized for analysis of DEG included Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA) and Cell type enrichment (Cten) software [34]. Transcripts with a FDR less than 0.05 and absolute LFC greater than 1 were used to generate z-scores and p-values in IPA. For Cten, DEG were converted from their Ensembl transcript ID to their associated gene name using Biomart [35], and then input into Cten.

For co-expression analysis, the Weighted Gene Co-expression Network Analysis (WGCNA) package in R was utilized. WGCNA clusters genes into modules based on expression levels and correlates variation in expression levels in those modules to traits of interest. Transcripts with less than four total counts across all samples were removed prior to normalization with DESeq2 [31] for the WGCNA analysis. A soft threshold of 13 was utilized to generate an adjacency matrix based on co-expression. Minimum module size was set to 30. Modules were then correlated to different traits. For discrete traits, including line, dpi, treatment, and sex, each chicken was given a nominal value of 2, 1, or 0. For the continuous traits, viral load was reported in log copy number and antibody load by sample-to-positive ratio. Viral load was obtained by qPCR of the chicken lachrymal fluid at 2 and 6 dpi, and an ELISA for NDV antibody was used to measure serum antibody levels at 10 dpi, as described previously [11].

Transcript IDs with FDR less than 0.05 were converted to associated gene names using BioMart on Ensembl [35]. For gene ontology (GO) analysis, associated gene names were input into the Generic GO term finder [36]. The chicken genome was selected and default parameters were used to generate significant GO terms associated with a set of genes.

The Fluidigm Biomark system (Fluidigm, South San Francisco, CA) was used to further validate the RNA-seq technology. The isolated RNA used to construct the cDNA libraries was also used as input for Fluidigm Biomark. The genes selected for this experiment were chosen to represent a range of fold changes across three tissues: trachea [11], lung, and Harderian gland (Lamont, personal communication). Primers were created based on the previous reference genome, Gallus_gallus-4.0 (Gal4; GCA_000002315.2), and the sequences for the 35 genes used in the current study were previously published [11]. Fluidigm Biomark HD $\log_2$ fold change (LFC) was calculated by the $-2^{-\Delta\Delta CT}$ method, utilizing *H6PD* as the housekeeping gene. In order to ensure the correct transcript ID was used for correlation to the Fluidigm data, the primer sequences were blasted against the current reference genome, Gal5. If the primers did not find a perfect match or if the matching transcript ID was not found in the edgeR output (likely removed due to low counts or during normalization process), that gene was excluded from the analysis. In two instances (*MHCI-likeY* and *CD40*), primers matched to two different transcript IDs, so the LFC calculated by edgeR for the two transcripts was summed prior to correlation with the Fluidigm Biomark HD LFC.

Results

RNA-seq output summary and lung viral load

Approximately 93% of the nearly 12 million filtered reads per sample mapped to the Gal5 reference genome (Table 3-2). The transcriptome coverage, percentage of transcripts with at least 1 count, was on average 38.96%. Using Gal5 increased the mapping percentage by 2% but decreased the transcriptome coverage when compared to the previous reference genome (Gal4), which is due to the addition of approximately 20,000 transcripts to the Gal5 reference genome. There appeared to be no major differences in the summary statistics of the

RNA-seq output (Table 3-2) between the treatment groups that could cause biases. The unmapped reads were further analyzed to attempt to detect viral transcripts as previously described [11], but no viral transcripts were detected in the unmapped reads of the lung.

Table 3-2: Summary statistics of RNA-seq output.

	Pre-filtered Reads	Filtered Reads	[a]Mapping %
Leghorn	15,068,576	12,506,656	92.81
Fayoumi	13,022,086	10,816,065	92.49
Nonchallenged	14,490,133	12,050,144	92.71
Challenged	13,636,113	11,303,680	92.60
All	14,045,331	11,661,361	92.65

[a]Percentage of filtered reads that mapped uniquely to the Gal5 reference genome.

Principal Component Analysis shows large impact of line and sex on lung transcriptome

The PCA plot generated from the 500 genes with the most variance in the lung transcriptome displayed a clear separation by line and sex (Figure 3-1). The first principal component (PC1) accounted for 41.28% of the variance in the lung transcriptome and corresponded to the line differences. The second principal component accounted for 13.78% of the variance, but did not correspond to any of design parameters (data not shown). The third principal component (PC3) accounted for 7.57% of the variance in the lung transcriptome and represented sex differences. Due to the large amount of variance accounted for by PC3, sex was incorporated into the edgeR model to determine which genes were differentially expressed (DE). Many genes found in the top and bottom loadings of the PCs are classified as lincRNA or uncharacterized proteins (Table 3-3). Interestingly, zinc finger protein 366 (*ZNF366*) contributes to the bottom loadings for both PC1 and PC3 and is located on the Z chromosome.

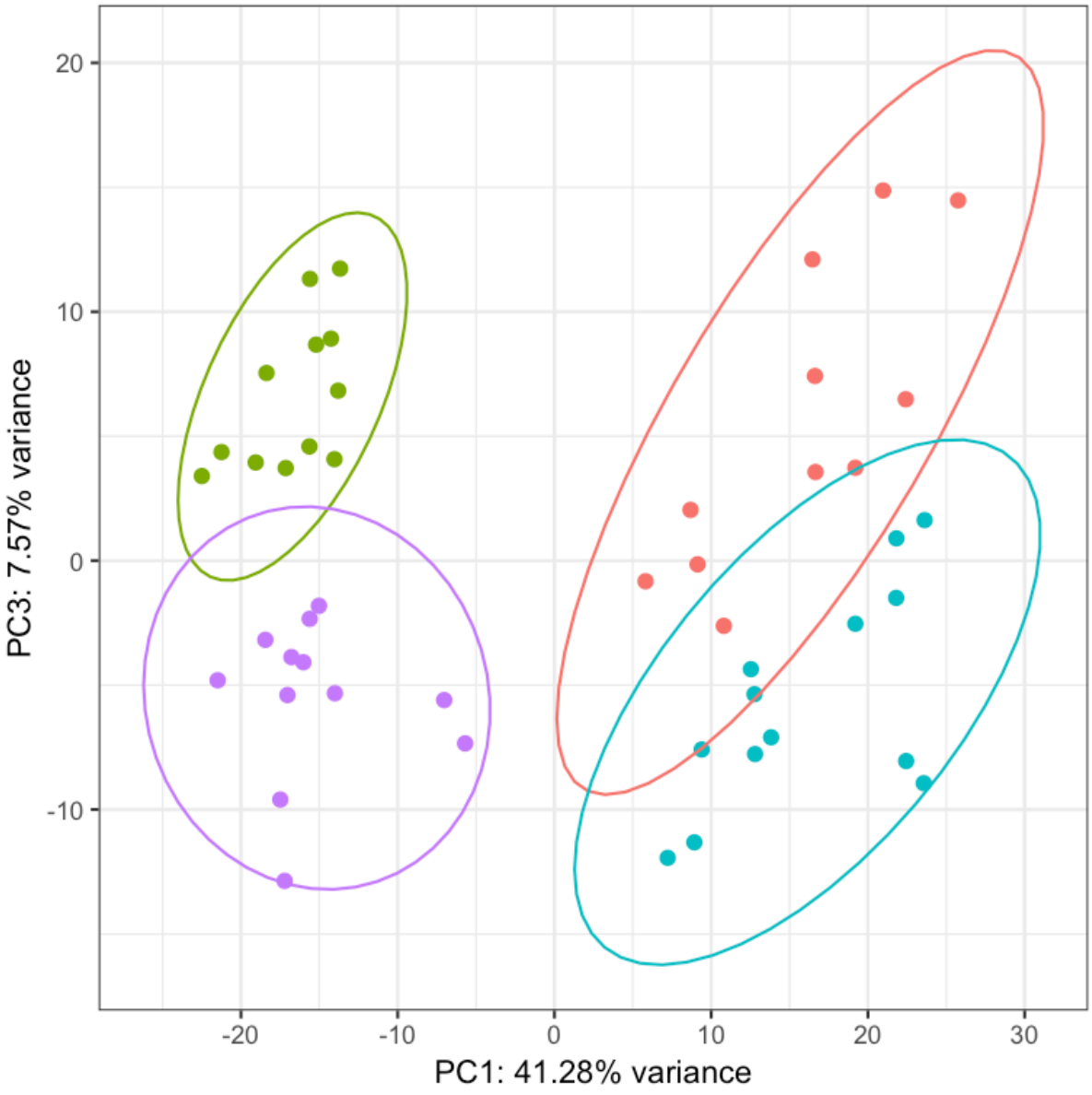

Figure 3-1: PCA plot suggests genetic line and sex account for large amounts of the variation. pcaExplorer generated principal component analysis (PCA) plot using the top 500 genes to display the variance associated with a principal component (PC). The samples (dots) are color labeled by sex and line: Fayoumi, female (pink), Fayoumi, male (blue), Leghorn, female (green), Leghorn, male (purple). The ellipses around each group are drawn with a confidence interval of 0.95.

Table 3-3: Top 10 top and bottom loadings for PC1 and PC3.

	ID (description)
Top loadings PC1	ENSGALT00000052188 (lincRNA) ENSGALT00000084582 (lincRNA) ENSGALT00000010657 (*SYT8, synaptotagmin 8*) ENSGALT00000085449 (lincRNA) ENSGALT00000074783 (lincRNA) ENSGALT00000084401 (lincRNA) ENSGALT00000082899 (*DPT, dermatopontin*) ENSGALT00000087260 (lincRNA) ENSGALT00000086210 (lincRNA) ENSGALT00000084683 (lincRNA)
Bottom loadings PC1	ENSGALT00000065772 (lincRNA) ENSGALT00000041171 (*CDHR1, cadherin related family member 1*) ENSGALT00000010186 (*LOC428958, Lipase*) ENSGALT00000026579 (*LINC00954, uncharacterized protein*) ENSGALT00000088678 (protein coding) ENSGALT00000023730 (*ANKRD55, ankyrin repeat domain 55*) ENSGALT00000010222 (*LOC424523, uncharacterized protein*) ENSGALT00000012370 (*LOC422316, uncharacterized protein*) ENSGALT00000008605 (*CA4, carbonic anhydrase 4*) ENSGALT00000024201 (*ZNF366, zinc finger protein 366*)
Top loadings PC3	ENSGALT00000067307 (protein coding) ENSGALT00000080873 (*RCJMB04_7i8, uncharacterized protein*) ENSGALT00000046430 (*faf, Female-associated factor FAF*) ENSGALT00000077789 (*Nipped-B homolog-like*) ENSGALT00000086634 (lincRNA) ENSGALT00000054249 (protein coding) ENSGALT00000056168 (protein coding) ENSGALT00000084245 (protein coding) ENSGALT00000085229 (lincRNA) ENSGALT00000050894 (protein coding)
Bottom loadings PC3	ENSGALT00000044087 (*ARRDC3, arrestin domain containing 3*) ENSGALT00000083633 (lincRNA) ENSGALT00000009519 (*IGLL1, uncharacterized protein*) ENSGALT00000024328 (*ACER2, alkaline ceramidase 2*) ENSGALT00000073167 (protein coding) ENSGALT00000024902 (*SLC44A1, solute carrier family 44 member 1*) ENSGALT00000024201 (*ZNF366, zinc finger protein 366*) ENSGALT00000012370 (*LOC422316, uncharacterized protein*) ENSGALT00000018840 (*JCHAIN, joining chain of multimeric IgA and IgM*) ENSGALT00000024747 (*PCGF3, polycomb group ring finger 3*)

Contrasting challenged and nonchallenged birds within each line and time

The gene expression of challenged and nonchallenged birds was contrasted within each time point and each line. These contrasts resulted in 16 and 101 DEG between the challenged and nonchallenged birds in the Leghorn and Fayoumi, respectively at 2 dpi. Two DEGs overlap between the Fayoumi and Leghorn in the contrast of challenged and nonchallenged at 2 dpi: *ZNFX1* and a novel transcript (ENSGALT00000021310). Of the 101 DEG at 2 dpi in the Fayoumis, some genes of interest included *CISH*, *LINGO1*, *TGFBI*, *C6*,

and *COL5A1*. The GO term analysis of these 101 genes resulted in no significant immune related GO terms, and most of the top GO terms were related to development, including single organism developmental progress, developmental process, and system development. For the 16 DEG at 2 dpi in the Leghorns, *C1QA*, *MARCO*, and *MPEG1* were immune genes of interest. At 6 dpi, there were no DEG due to treatment in the Fayoumis, but there were 2 DEG in the Leghorns: Metazoa_SRP and unknown. At 10 dpi, there were no DEG due to treatment in the Leghorns. Overall, the limited number of DEG in the Leghorn suggests non-responsiveness to NDV challenge in the lung. In the Fayoumis, 2,537 DEG were identified when contrasting the challenged vs. nonchallenged at 10 dpi.

Several pathways were significantly impacted by the challenge at 10 dpi in the Fayoumis, as predicted by Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA) (Figure 3-2). Overall, many of these pathways lead to cytoskeleton regulation and cell proliferation/death/movement. In particular, gene expression in the Ephrin B Signaling pathway was predicted to activate cell proliferation, dendrite remodeling, cytoskeleton regulation, and development of focal adhesions. Thrombin signaling was predicted to activate protein synthesis, cell survival, and platelet aggregation. Expression levels in the IL-8 Signaling pathway result in the predicted activation of mobilization of $Ca2^{+}$, exocytosis, endothelial cell migration, endothelial tube formation, angiogenesis, inflammation, cell adherence, neutrophil degranulation, superoxide production, adherence of neutrophils and monocytes, and inhibition of endothelial cell retraction. mTOR signaling results in activated autophagy regulation and actin organization and inhibition of translation. EIF2 Signaling was inhibited, however this pathway was more activated in the nonchallenged Fayoumis than the Leghorns at 10 dpi (data not shown).

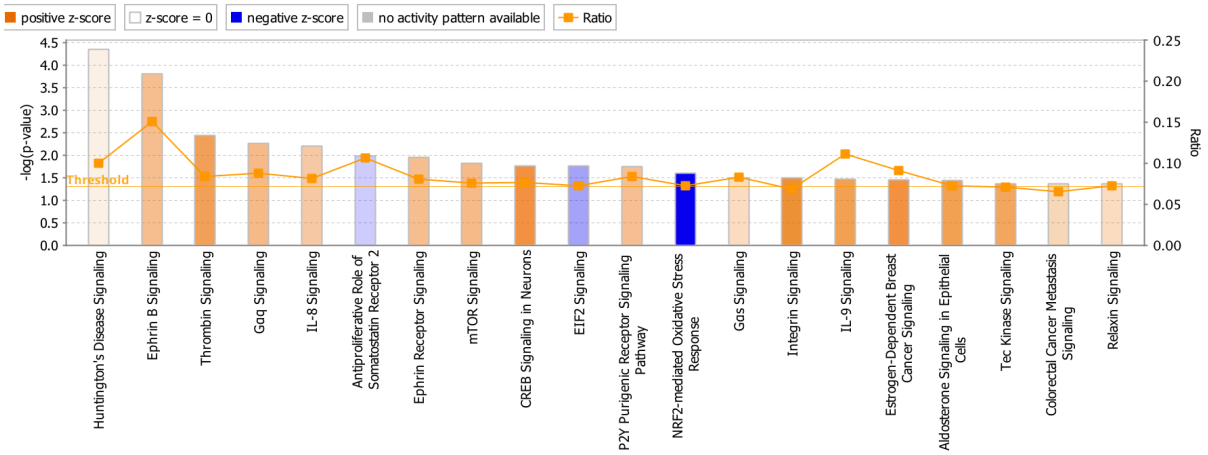

Figure 3-2: Top canonical pathways in the transcriptome of challenged Fayoumis at 10 dpi. The expression values of transcripts with an absolute $\log_2$ fold change (LFC) > 1 and a false discovery rate (FDR) < 0.05 were used to calculate the z-scores and –log(p-values) for each pathway. Pathways shown in this figure have an absolute z-score>0.01 and a –log(p-value)>1.3. Pathways represented are predicted to be either activated (orange) or inhibited (blue). The more intense these colors, the higher the absolute z-score. The height of each bar corresponds to the –log(p-value). The ratio (orange line) represents the proportion of genes within the pathway that were DE.

Contrasting the Fayoumis and Leghorns within each treatment group and time

Although there were relatively few differences between the challenged and nonchallenged birds (with the exception of Fayoumis at 10 dpi), we do see evidence of impact of the challenge on gene expression in the lung when the lines were directly compared (Figure 3-3). There were 233 genes consistently DE between Fayoumi and Leghorn regardless of time or challenge, and all of these agreed in LFC direction across contrasts. A total of 441, 512, and 336 genes were shared by the challenged and nonchallenged birds at 2, 6, and 10 dpi, respectively. Of these overlapping genes (DE in both challenged and nonchallenged, Fayoumi vs. Leghorn at each time), all were in agreement in LFC direction, with one exception: at 10 dpi, ENSGALT00000062549 was more highly expressed in the challenged Leghorns, but more highly expressed in the nonchallenged Fayoumis. This novel lincRNA, was also significantly impacted by the challenge*line interaction at 10 dpi.

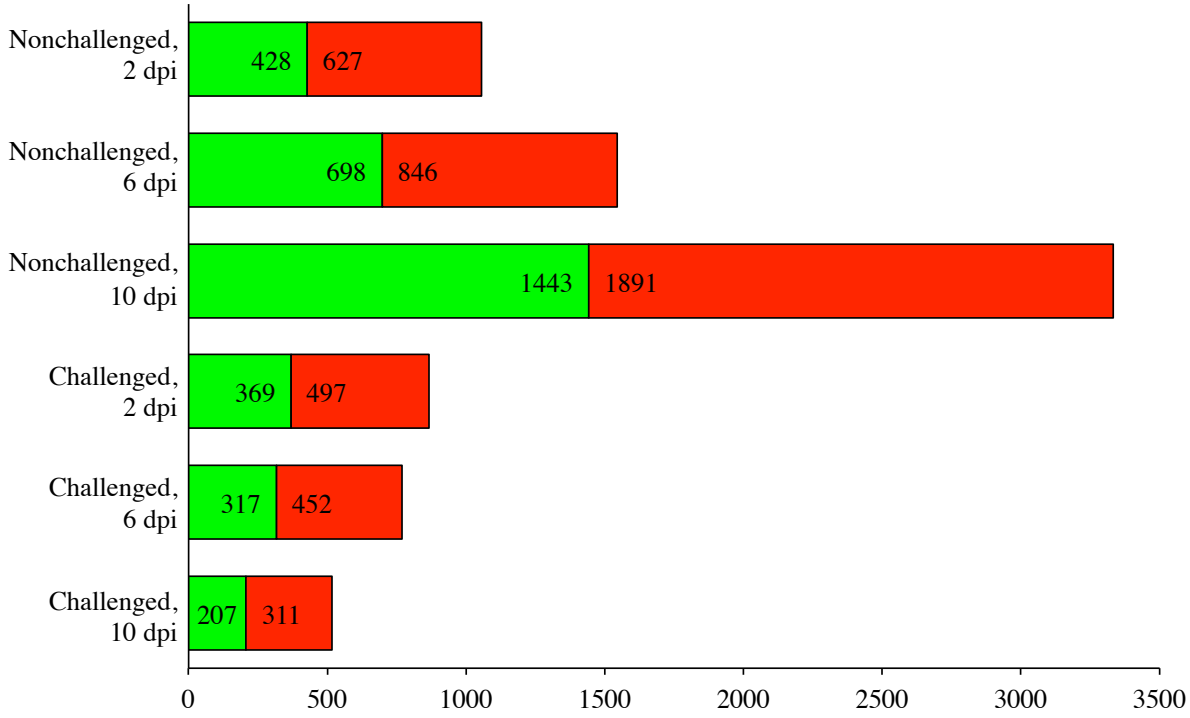

Figure 3-3: Differentially expressed genes between the Fayoumi and Leghorn at each time within each challenge state. The number of differentially expressed genes (FDR<0.05) between the Fayoumi and Leghorns are shown for the nonchallenged and challenged birds at each time point. Genes in green were more highly expressed in the Leghorns. Genes in red were more highly expressed in the Fayoumis. The number of genes more highly expressed in each line is labeled within the green and red bar, for each contrast.

The interaction between challenge and line at 10 dpi

At 2 dpi, only one gene was significantly impacted by both challenge and line at 2 dpi (*NOV-201*), and no genes were impacted by the interaction at 6 dpi. The 131 genes that were significantly impacted by both challenge and line at 10 dpi were further analyzed using STRING network analysis (Figure 3-4). A total of 83 nodes and 48 edges were incorporated, which resulted in an average node degree of 1.16 and average local clustering coefficient of 0.305. This network had significantly more interactions than expected (p=4.09e-06) and was significantly associated with the KEGG pathway, protein processing in endoplasmic reticulum (FDR=0.00838). The genes in this network are highly involved in protein translation and alternative splicing and may be important in antibody production.

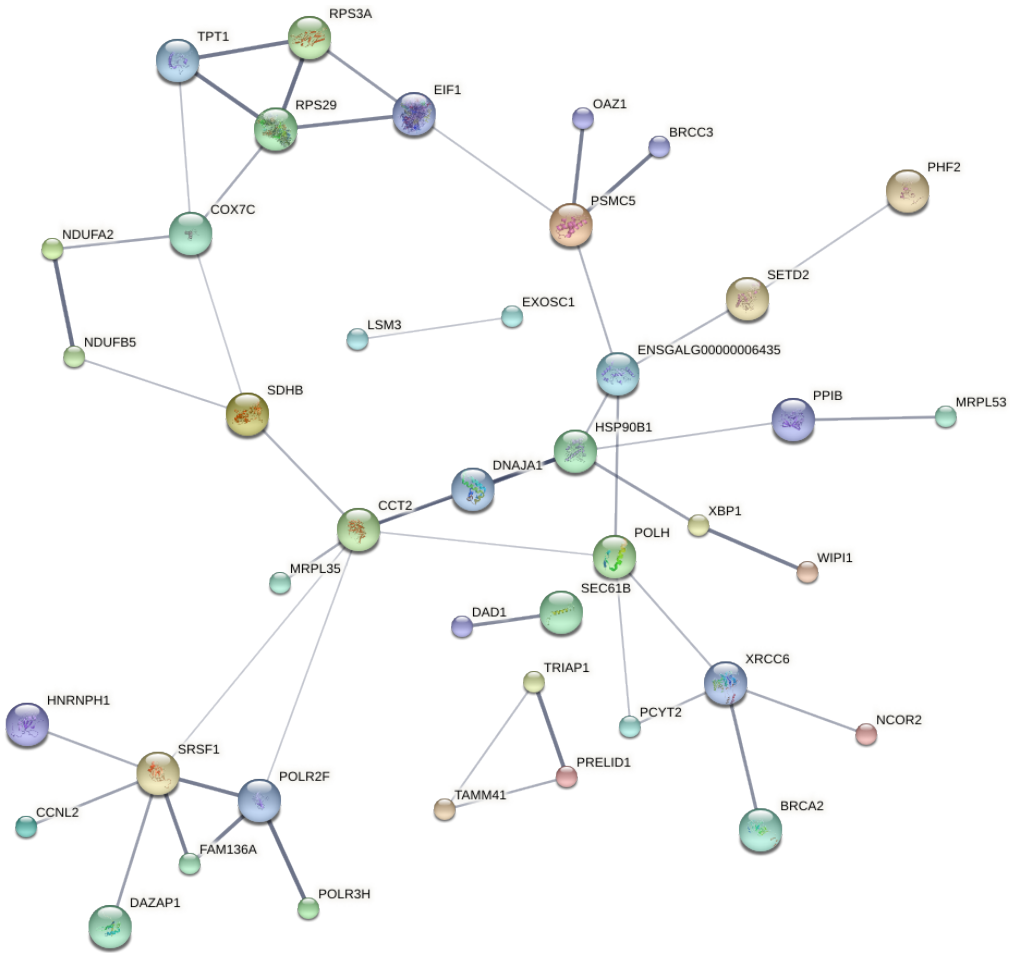

Figure 3-4: STRING network for genes impacted by the interaction between challenge and line at 10 dpi. Differentially expressed genes (FDR<0.05) from the challenge*line interaction at 10 dpi were input into STRING for network analysis. Disconnected nodes were removed and a medium confidence score (0.400) was utilized. All edges represent protein-protein interactions; the thicker the edge the more evidence to support that connection. Proteins of unknown structure (small nodes) and predicted structure (large nodes) are represented in various colors.

Temporal differences in the lung of nonchallenged Fayoumis and Leghorns

Days post infection appeared to impact the number of DEG, as there were fewer DEG between challenged Fayoumis and Leghorns as time progressed, but there were more DEG between the nonchallenged Fayoumis and Leghorns as time progressed (Figure 3-3). The increase in DEG over time in the nonchallenged birds could be related to developmental changes that differ between lines.

Temporal changes in the lung transcriptome are evidenced by high numbers of DEG when the time points were compared within line and treatment group (Figure 3-5). Overall, the nonchallenged Fayoumis showed the most changes over time in numbers of DEG. There were 246 DEG between the nonchallenged Fayoumis at 6 and 2 dpi and 441 DEG between 10 and 2 dpi. Only 145 of the DEG were shared between 6 and 2 dpi and 10 and 2 dpi, suggesting a complex developmental progression. When considering all DEG across time in the nonchallenged Fayoumis, several were immune related. Top GO terms associated with the DEG between 10 and 2 dpi in the nonchallenged Fayoumi included system development, neutrophil chemotaxis, animal organ development, and leukocyte migration, suggesting a close link between developmental changes and immune related changes in the Fayoumi lung over time.

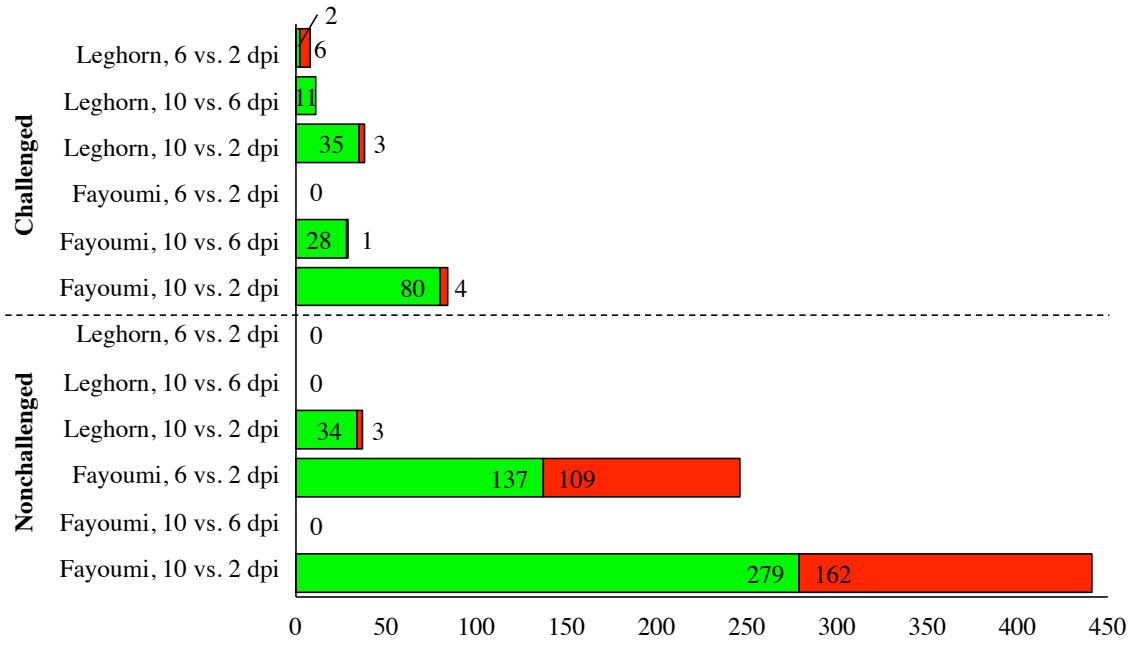

Figure 3-5: Temporal changes in gene expression within line and treatment group as measured by DEG. Each contrast (y-axis) compares two time points (2, 6, or 10 dpi), within each line (Fayoumi or Leghorn), and within each treatment group (challenged or nonchallenged). The dashed horizontal line separates the challenged birds (top) and nonchallenged birds (bottom). Transcripts more highly expressed in the earlier time point are green and those more highly expressed in the later time point are in red. Numbers within the bar chart correspond to the number of differentially expressed genes.

Changes in gene expression over time may be related to different composition of cells present in the lung. Cell type enrichment (Cten) software [34] predicts enriched cell types based on DEG. The genes that were more highly expressed at 2 and more highly expressed at 10 dpi in the nonchallenged Fayoumis were input into Cten separately for cell type enrichment analysis (Figure 3-6). At 2 dpi, a variety of cell types were significantly enriched, including uterus, adipocyte, smooth muscle, cardiac myocytes, fetal lung, and more (Figure 3-6A). At 10 dpi, nearly all significantly enriched cell types were immune related, such as lymph node, bone marrow, whole blood, thymus, lung, tonsil, and several specific immune cell types (Figure 3-6B). Although chickens do not have lymph nodes, this result may suggest an increase in the bronchus-associated lymphoid tissue of the lung. Between 23 and 31 days of age, the Fayoumi lung appears to be developing its immune competency by increasing the amount of immune related cell types.

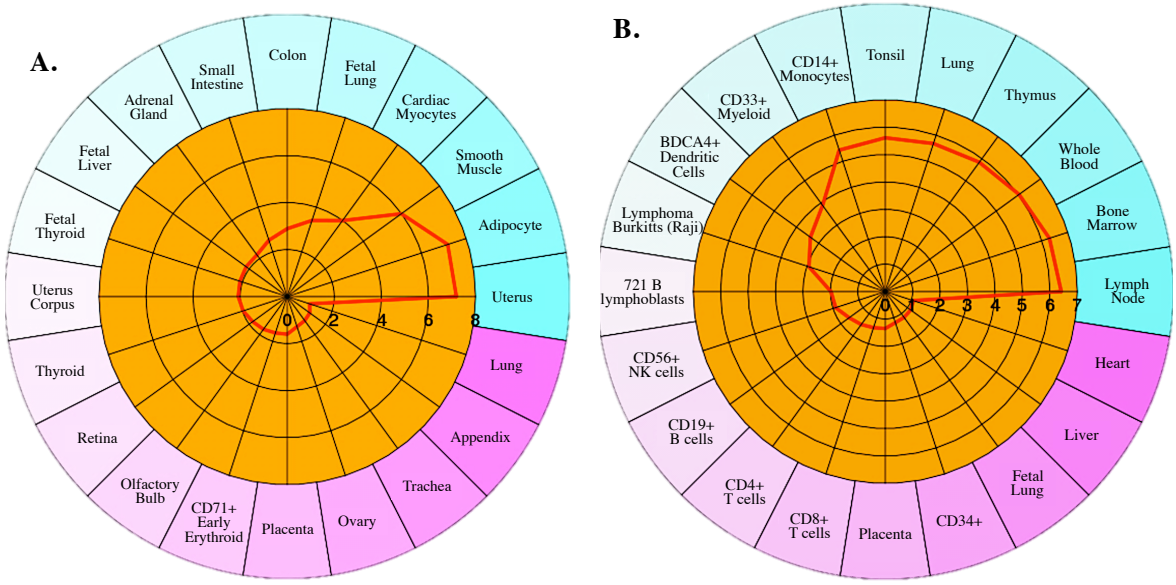

Figure 3-6: Comparing enriched cell types at 2 and 10 dpi of the nonchallenged Fayoumis. Tissue types (labeled outside circle) with an enrichment score (red line) greater than 2 were considered significant. A. The transcripts more highly expressed at 2 dpi in the nonchallenged Fayoumis were converted to their associated gene name and input into Cten. Of the 279 differentially expressed transcripts, 187 had an associated gene name, and Cten recognized 126. B. The transcripts more highly expressed at 10 dpi in the nonchallenged Fayoumis were converted to their associated gene name and input into Cten. Of the 162 differentially expressed transcripts, 95 had an associated gene name, and Cten recognized 69.

In the nonchallenged Leghorns, only 37 genes were DE between 10 and 2 dpi, and 0 genes were DE between the other time points (Figure 3-5). GO term analysis on the 37 DEG between 10 and 2 dpi resulted in 2 significant terms: oxygen transport and gas transport. Temporal differences in gene expression were unique to each line. These differences between lines may account for the increase in DEG between nonchallenged Fayoumis and Leghorns as time progressed.

Different DEG identified over time in the challenged Fayoumis and Leghorns

In the challenged Fayoumis, there were fewer DEG across time overall and the increase in DEG with time seemed to be delayed when compared to the nonchallenged Fayoumis (Figure 3-5). Most genes were more highly expressed at the earlier time point in the challenged Fayoumis.

The number of DEG in the challenged Leghorns appeared to be similar to the nonchallenged Leghorns, but the DEG between 10 and 6 dpi and 6 and 2 dpi in the challenged birds were not differentially expressed in the nonchallenged birds. The Leghorns had similar numbers of DEG between 10 and 2 dpi, in the challenged and nonchallenged birds, 38 and 37, respectively (Figure 3-5). However, only 1 DEG was shared between these two contrasts (ENSGALT00000007669). The DEG in the challenged birds may not be related to developmental changes and could be a result of the challenge. Only 22 DEG were shared between the challenged and nonchallenged 10 vs. 2 dpi contrasts in the Fayoumis.

Gene co-expression analysis

Due to the large differences in the nonchallenged birds over time and the unexpected numbers of DEG obtained through contrasts over time, WGCNA, which does not rely on contrasts, was utilized. All transcripts and their normalized count values were clustered into modules based on similarities in gene expression (Figure 3-7A). Those modules were then

correlated with traits of interest (Figure 3-7B). GO term analysis was performed to determine the function of genes within modules of interest. Several modules were significantly associated with immune related GO terms. The blue module genes were positively correlated with line, but were not significantly correlated with any other trait measured (dpi, treatment, sex, viral load at 2 and 6 dpi, and antibody levels at 10 dpi). Immune related GO terms that were significantly associated with these genes include defense response, regulation of response to stress, and regulation of inflammatory response. The genes of the black module were positively correlated with line and negatively correlated with treatment, viral load, and antibody levels. Viral life cycle, viral process, cellular response to stress, symbiosis, encompassing mutualism through parasitism, regulation of viral process, and response to stress were all immune related GO terms that were significantly associated with the black module. The skyblue3 module followed a similar pattern in module-trait correlations as the black module, except that skyblue3 module was negatively correlated with dpi was not associated with viral load at 6 dpi. Genes in the Skyblue3 module were significantly associated with integrin-mediated signaling pathway, positive regulation of apoptotic process, positive regulation of programmed cell death, positive regulation of cell death, positive regulation of apoptotic signaling pathway, and response to stress. Genes in the lightyellow module were strongly positively correlated with sex and negatively correlated with antibody levels. Most of the 224 genes in this module were located on the Z chromosome (80%), 4% on the W chromosome, 2% were autosomal, and 14% on scaffolds. The latter may belong to a sex chromosome. Genes within the lightyellow module were also significantly associated with cellular process, metabolic process, and single-organism process.

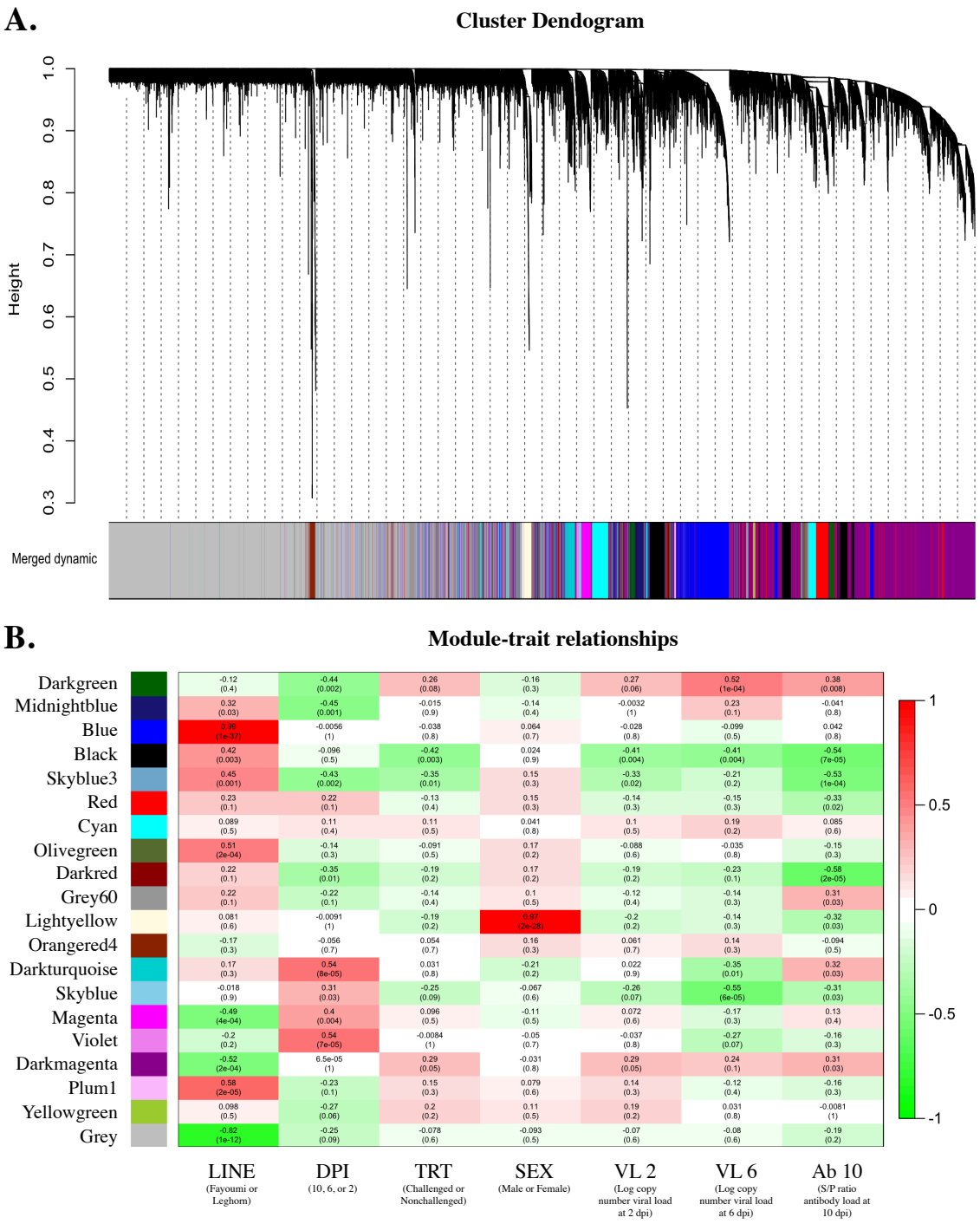

Figure 3-7: Cluster dendogram and module-trait relationships from WGCNA. A. Cluster dendogram shows the transcript relationships and their corresponding module. Each color represents a module. B. Each module (y-axis) is correlated to each phenotype (x-axis) the correlation and p-values were reported for each comparison. Strong positive correlations are colored in red, and strong negative correlations, green. Discrete factors were given a value of 0, 1, or 2. The factor given the higher value is listed first underneath the trait, in parenthesis. The units of the quantitative traits (VL 2, VL 6, and Ab 10) are given underneath the trait in parenthesis. Days post infection (DPI); Treatment (TRT); Viral load (VL); Antibody (Ab)

Driver genes within modules of interest were examined

Within each module, genes with high absolute gene significance for a trait and high absolute module membership are likely critical hub genes for the module. The top three transcripts, based on highest absolute gene significance, for the module with the strongest correlation for each trait were identified (Table 3-4).

Table 3-4: Top 3 transcripts for the top modules for each trait.

Trait	Module	Top 3 Transcripts (description)	(GS[a], MM[b])
Line	Blue	ENSGALT00000052188 (lincRNA)	(0.976, 0.964)
		ENSGALT00000087010 (protein coding)	(0.976, 0.966)
		ENSGALT00000066798 (lincRNA)	(0.974, 0.962)
[c]DPI	Darkturquoise	*RNF208* (Ring finger protein 208)	(0.644, 0.753)
		LAMA2 (Laminin subunit alpha 2)	(-0.644, -0.562)
		LCK (LCK proto-oncogene, Src family tyrosine kinase)	(0.643, 0.74)
Treatment	Black	*cNFI-A* (Nuclear factor 1 C-type)	(-0.671, 0.694)
		RHOT2 (Ras homolog family member T2)	(0.61, -0.827)
		UBE2E3 (Ubiquitin conjugating enzyme E2 E3)	(-0.576, 0.812)
Sex	Lightyellow	ENSGALT00000077789 (Nipped-B homolog-like)	(-0.987, -0.937)
		ENSGALT00000085229 (lincRNA)	(-0.985, -0.94)
		ENSGALT00000080994 (protein coding)	(-0.984, -0.964)
2 dpi viral load	Black	*cNFI-A* (Nuclear factor 1 C-type)	(-0.648, 0.694)
		RHOT2 (Ras homolog family member T2)	(0.596, -0.827)
		CDC5L (Cell division cycle 5 like)	(-0.586, 0.681)
6 dpi viral load	Skyblue	*FAM98B* (Family with sequence similarity 98 member B)	(-0.609, 0.777)
		FUT8 (Alpha-(1,6)-fucosyltransferase)	(-0.605, 0.87)
		ATP6V1H (V-type proton ATPase subunit H)	(-0.592, 0.849)
10 dpi antibody level	Darkred	ENSGALT00000068419 (protein coding)	(-0.754, 0.895)
		FSTL1 (Follistatin like 1)	(-0.729, 0.844)
		NOV (Nephroblastoma overexpressed)	(-0.719, 0.794)

[a]Gene Significance (GS); [b]Module Membership (MM); [c]Days post infection (DPI)

Many of the top transcripts associated with these traits have limited functional information available, especially functional information specific to chickens. The top transcripts associated with line in the blue module were novel, with no associated gene names. ENSGALT00000052188 was also a top-loading gene for PC1 (Table 3-3).

Of the transcripts associated with sex, two of the three genes are located on the W chromosome and the other (ENSGALT00000080994) on a scaffold; it is possible this scaffold should be placed on the W chromosome. ENSGALT00000077789 and ENSGALT00000085229 were also top loading genes for PC3 (Table 3-3). Several novel associations were made between driver genes and traits of interest in this study. Further

investigation of the impact of these driver genes on other traits can help elucidate the overall function of the protein.

Fluidigm Biomark was utilized to validate the RNA-seq results

The RNA-seq results were validated using the Fluidigm Biomark system. Although several genes had low LFC and clustered around zero, the correlation of LFC between the two methods was high (r=0.82) (Figure 3-8).

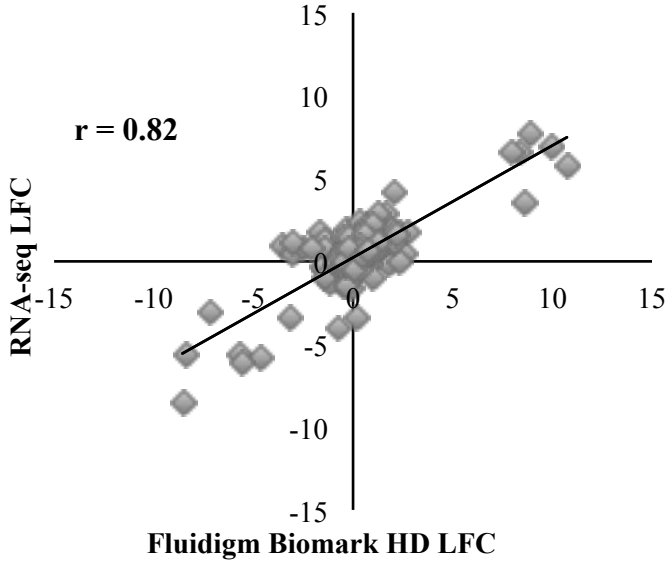

Figure 3-8: Validation of RNA-seq technology using the Fluidigm HD Biomark system. This plot is composed of 210 points comparing the log2 fold change (LFC) as estimated by Fluidigm Biomark HD (x-axis) and RNA-seq (y-axis) for the Fayoumi vs. Leghorn contrasts within each time (2, 6, 10 dpi) and each treatment group (challenged, nonchallenged), resulting in six total contrasts for 35 genes.

Discussion

The lung plays a crucial role in the immune system of chickens, especially after challenge with respiratory pathogens. The BALT, located in the lung, is home to B and T cells and can impact the clinical outcome of disease [20]. No detectable virus was found in the lung at the time points measured; however, due to the high virus levels detected in the trachea [11], the respiratory nature of lentogenic NDV [17], and the lung's importance in local immunity, examining the lung transcriptome was valuable.

Sex had a large impact on lung transcriptome

There were surprisingly large sex effects in a non-sex related organ in young chickens, likely due to incomplete dosage compensation [37-39]. The lightyellow module, which was strongly positively correlated with sex, included 179 transcripts from the Z chromosome, which is approximately 10% of the total number of transcripts from that chromosome. The overlap of top and bottom loading genes identified in the PCA with driver genes identified from the lightyellow module give strong confidence to the impact of sex on these genes.

Sex differences have previously been related to different disease outcomes in birds [40-42]. Nipped-B homolog-like, ENSGALT00000077789, a driver gene associated with sex in the lightyellow module, was previously associated with hemoglobin levels in mammals [43]. Previous studies have also pointed to the importance of hemoglobin genes in disease resistance to AIV [15]. The lightyellow module was also negatively correlated with antibody levels. Future RNA-seq studies in the chicken should balance and account for sex in their design.

NDV stimulated a large number of DEG in the Fayoumi at 10 dpi

The large number of NDV-induced DEG at 10 dpi in the Fayoumis strongly suggests unique responses to NDV between the lines. The mTOR signaling pathway was significantly activated in challenged Fayoumis at 10 dpi and based on expression levels was predicted to result in autophagy. Increased autophagy benefits NDV replication [44]. EIF2 signaling, which can lead to inhibition of NDV replication [45], was downregulated in the challenged Fayoumis at 10 dpi, but was more activated in the Fayoumis than Leghorns at 10 dpi in the nonchallenged birds. Previous comparisons have shown this pathway to be more activated in Fayoumi than Leghorn in the trachea and spleen of challenged birds at 2 dpi [11] (Lamont,

personal communication). This pathway results in inhibition of viral replication through inhibition of translation and increased apoptosis [45]. Reduced autophagy and increased apoptosis would be beneficial in cells that are infected with the virus, however, NDV was not detectable at any of the time points in the lung transcriptome. The absence of detectable virus in the lungs of these challenged birds alters the strategy used by the host to defend itself from the virus.

Although the specific reason for the relatively low number of DEG between challenged and nonchallenged birds of both lines at the 6 dpi time is not clear, it may relate to a period of transition from innate to acquired immune mechanisms. Immune response as measured by GO term analysis of DEG in the lung was detected as early as 6 dpi in six-week-old birds after challenge with a lowly pathogenic AIV [46]. Different pathogens and ages of the host may cause different response timelines in the lung.

The transcriptomic response in the lung to NDV was unique when compared to the response of the tracheal epithelial cells of the same birds [11]. The number of DEG between challenged and nonchallenged birds decreased over time in the trachea [11]. In the lung the delayed response in the Fayoumi and non-responsiveness in the Leghorn may be due to the lack of detectable virus, while high levels of virus were detected in the trachea of these birds [11]. In contrast, in a separate experiment with commercial Leghorns, 4 days after challenge with AIV a higher viral load was observed in the lung than trachea [47].

Based on gene expression in the lung, 10 dpi appears to be a crucial time in the immune response timeline for the Fayoumis. Genes that were impacted by both challenge and line are of particular interest in the search for genes related to disease resistance. One of these genes is *PPIB,* also known as cyclophilin B, which is a chaperone protein involved in

modulating the host immune response [48]. Previous reports have shown that *PPIB* can increase the rate of IgG folding [49-51]. Further investigation into this protein's role in chickens and in response to NDV is necessary.

A conserved response to this virus by the two lines, as evidenced by the fewer DEG between the challenged Leghorns and Fayoumis as time progressed, may suggest a slow ramp-up of the immune response in the lung. A previous study [15] also found more DEG between nonchallenged Fayoumis and Leghorns than between the AIV challenged Fayoumis and Leghorns in the lung. It is possible that gene expression in the Fayoumi and Leghorn becomes less different after an immune challenge because the two lines are marshaling similar mechanisms of response in the lung. However, these two lines are known to differ in their response to multiple pathogens.

Genetic selection based on gene expression

The black module genes' expression levels negatively correlated with viral load and were positively correlated with line. The blue module genes were strongly positively correlated with line, but were not correlated with the other immune traits measured; nonetheless, they were immune related as evidenced by GO term analysis. The basal expression levels of these genes may be important to the relative resistance of the Fayoumi to other pathogens. In practice, it is important not to select on one phenotype or for resistance to one pathogen, as this may increase susceptibility to another pathogen. Selection based on basal gene expression levels has been successful [52]. Genes in the black and blue modules may be good candidates for selection.

NDV may have impacted normal lung development

Differences between the two lines were also seen in the number of DEG between the multiple time points. A previous study observed an impact of genetic line on lung

development in the transcriptomes of mice, which included differences in immune system chemotaxis, neurogenesis, and the extra-cellular matrix composition [53]. The increased DEG seen across time in the nonchallenged Fayoumis compared to the challenged Fayoumis and GO term analyses, unveiled a relationship between the challenge and developmental changes in the lung transcriptome related to the immune system and development. This was likely due to resource allocation.

The fewer DEG between time points in the challenged Fayoumis could be a direct result of the NDV challenge and resource allocation, because their energy was utilized to mount an immune response and, therefore, growth and development were arrested. Of the top driver transcripts associated with traits of interest, *RHOT2*, *ATP6V1H*, ENSGALT00000068419, and *FSTL1* all have associations with weight and growth related traits [54-57]. Repeated appearance of growth related associations among the top driver genes identified by WGCNA provide further evidence that developmental traits may be impacted by NDV challenge.

WGCNA identified potentially important driver genes

LAMA2 decreased in expression over time and is associated with collagen and bacterial invasion [58] and important for CD4+ CD8+ immature thymocytes in mice [59]. The relationship between collagen and immune cell development was previously identified as a potential mechanism of resistance to NDV in the Fayoumi [11]. *LCK* increases in expression over time and is known to be involved in T cell receptor signaling. The expression levels of genes within the darkturquoise module were also negatively correlated with viral load at 6 dpi and positively correlated with antibody levels at 10 dpi. Previous studies have shown the importance of cell mediated immunity in host defense against NDV, but cell

mediated immunity is not sufficient to protect the host, as antibodies are also important for lasting immunity [60, 61]. These genes are likely critical to the chicken's overall response to NDV, regardless of genetic background.

Conclusions

Although no virus was detected in the lung, the reported results are still of value in observing how the lung tissue responds when the animal is infected. Transcriptional profiling in a tissue may reveal a response to events that occur at earlier times in the same tissue, or at remote sites in the body (communicated via circulating cells or molecules). Line and sex each had a large impact on the lung transcriptome as seen by PCA and WGCNA. The two lines responded uniquely to NDV, which offers insights into their mechanisms of resistance and susceptibility. The challenge may have interrupted normal lung development, which was also different between the two lines, as measured by numbers of DEG between the time points. Genes that were significantly impacted by the interaction between challenge and line at 10 dpi, especially *PPIB*, may play a critical role in antibody production. The response of the lung transcriptome was distinctly different from that of the tracheal epithelial cells of the same birds [11]. This highlights the value of examining multiple tissues and time points to more comprehensively understand the mechanisms of disease resistance in chicken. Future studies should examine protein expression levels, immunohistochemistry, and gene knockouts and their response to multiple strains of NDV to confirm true mechanisms of resistance.

Author contributions

MSD: Collected samples, isolated RNA, constructed cDNA libraries, processed and analyzed RNA-seq data, wrote paper
RAG: Experimental design, prepared viral isolate for inoculation, reviewed and edited paper
DAB: Experimental design, reviewed and edited paper
JCMD: Experimental design, advised on statistical analyses, reviewed and edited paper

HZ: Experimental design, reviewed and edited paper
SJL: Experimental design, reviewed and edited paper, oversaw analysis of data

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CHAPTER 4. NOVEL ANALYSIS OF THE HARDERIAN GLAND TRANSCRIPTOME RESPONSE TO NEWCASTLE DISEASE VIRUS IN TWO INBRED CHICKEN LINES

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Abstract

Behind each eye of the chicken resides a unique lymph tissue, the Harderian gland, for which RNA-seq analysis is novel. We characterized the response of this tissue to Newcastle disease virus (NDV) in two inbred lines with different susceptibility to NDV across three time points. Three-week-old relatively resistant (Fayoumi) and relatively susceptible (Leghorn) birds were inoculated with a high-titered La Sota strain of NDV via the eyes and nares. At 2, 6, and 10 days post infection (dpi), birds were euthanized and their Harderian glands were collected and analyzed via RNA-seq. The Fayoumi had significantly more detectable viral transcripts in the Harderian gland at 2 dpi than the Leghorn, but cleared the virus by 6 dpi. At all three time points, few genes were declared differentially expressed (DE) between the challenged and nonchallenged birds, except for the Leghorns at 6 dpi, and these DE genes were predicted to activate an adaptive immune response. When compared to the Leghorn, the Fayoumi was predicted to activate more immune pathways in both challenged and nonchallenged birds suggesting a more elevated immune system in the

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Fayoumis. Overall, this study helps to characterize function of this important but poorly characterized tissue and its response to NDV.

Introduction

The Harderian gland is a small tissue located behind the eyes of the chicken. In adult chickens, the average weight was found to be 84.4 mg [1]. The major functions of this tissue have not been fully characterized but include lubrication of the nictitating membrane [2] and several immune related tasks [3]. The cells of the Harderian gland are both bursa and thymus dependent [4,5]. Large numbers of heterophils and plasma cells accumulate in the chicken Harderian gland by two weeks of age [2], and this is a location of terminal B cell maturation [6]. Antigenic stimulation of the Harderian gland also increases the number of plasma cells [7,8]. Although some studies have shown evidence of low numbers of T cells residing in the Harderian gland [9,10], others saw an increase in T cell numbers after infection with Newcastle disease virus (NDV) [11,12]. There have been several studies on this unique tissue in the past 50 years, but there has been very little recent characterization of the Harderian gland with new technologies. To the authors' knowledge, the Harderian gland transcriptome has never been reported prior to this study.

NDV is a major global problem in poultry. This virus can cause high levels of mortality when birds are not vaccinated, or vaccinated improperly [13]. Eye-drop vaccination is a common method and it is unknown how the transcriptome of the closest immune tissue responds to vaccination.

Genetics plays a role in the chicken's response to NDV [14-16] and to the live virus vaccine [17-19]. A previous study has identified two inbred chicken lines that differ in their susceptibility to NDV [17]. Studying the response of the Fayoumi (relatively resistant to NDV)

and Leghorn (relatively susceptible to NDV) Harderian gland can offer insights into both vaccine development and host resistance. We predict these two lines will respond differently to NDV in the Harderian gland transcriptome in terms of viral transcript counts, numbers of differentially expressed genes (DEG), and activated pathways. These differences will help to identify possible mechanisms of NDV resistance in the chicken.

Results

Summary statistics of the RNA-seq reads

Overall, no bias was shown due to sequencing or mapping differences among groups (Table 4-1). Each sample was composed of two technical replicates, for which the raw counts for each transcript were summed. The average transcriptome coverage percentage, i.e. the percentage of transcripts with at least 1 count, was 37.0%, nearly 2% lower than that of the lung [18]. The percentage of reads that mapped to the reference genome on average across all samples was 91.17% (Table 4-1).

Table 4-1: Summary Statistics of RNA-seq reads

	Raw Reads	Filtered Reads	Mapping %
Average	26,110,910	21,274,532	91.17
Leghorn	23,376,646	18,941,376	91.42
Fayoumi	28,964,055	23,709,129	90.91
Challenged	26,960,703	21,908,635	91.13
Nonchallenged	25,224,170	20,612,859	91.21

Viral sequences were detected in the unmapped reads of challenged birds

The main effects of line ($p<0.0001$), dpi ($p<0.0001$), and viral gene ($p=0.015$) all had a significant impact on the viral transcript counts per million reads (cpm) (Figure 4-1). There was also a significant interaction between line and dpi ($p<0.0001$). The Fayoumis at 2 dpi had significantly higher viral transcript cpm than the Fayoumis or Leghorns at any time

point. Numerically, the Leghorns continued to have more detectible viral transcript counts at 6 dpi, whereas the Fayoumi had no detectable viral transcript counts at this time (Figure 4-1).

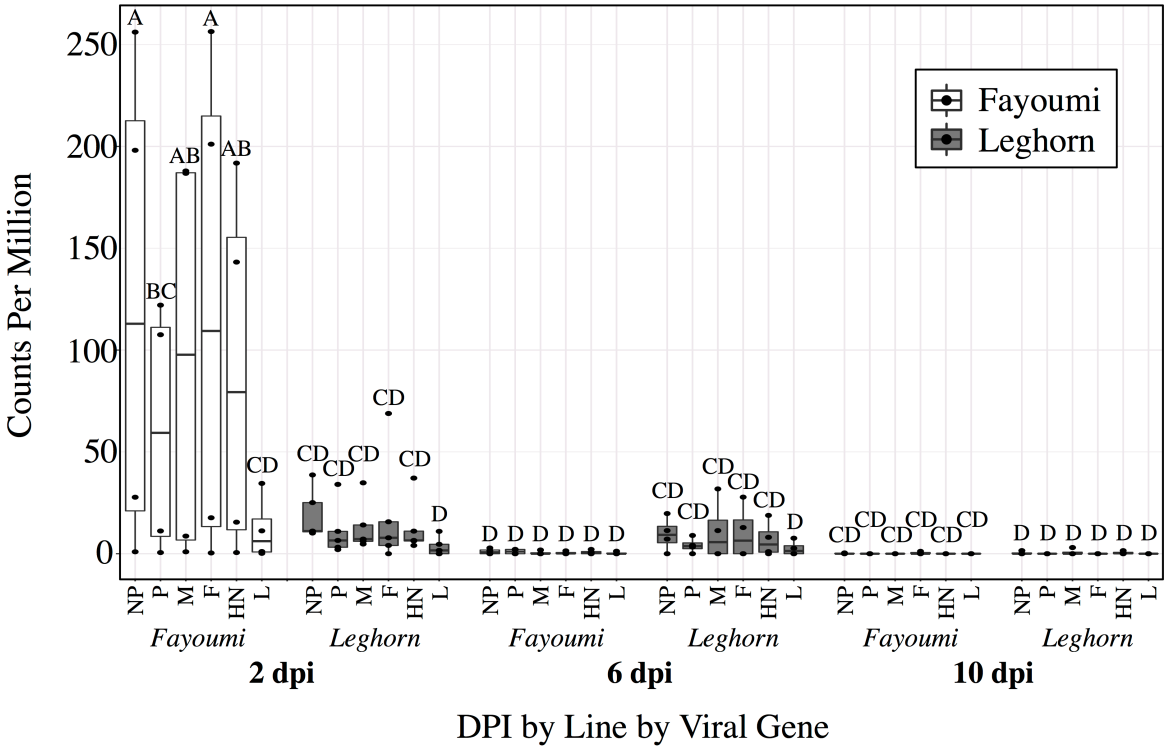

Figure 4-1: Viral transcripts detected in the unmapped reads of the challenged Fayoumis and Leghorns at 2, 6, and 10 days post infection. Points within each box plot represent the counts per million (cpm) (y-axis) of each viral gene from 3' to 5': Nucleoprotein (NP), Phosphoprotein (P), Matrix protein (M), Fusion protein (F), Hemagglutinin-neuraminidase (HN), or Polymerase (L), in the challenged Fayoumi (white) or Leghorn (grey), at 2, 6, or 10 dpi (x-axis). A Student's t-test connecting letters report was generated. Box plots that do not share letters are significantly different from each other. For each group n=4, except the Leghorns at 2 dpi (n=5) and the Fayoumis at 10 dpi (n=3).

The principal component analysis plot shows clear clustering by line

The principal component analysis (PCA) plot generated by pcaExplorer shows that principal component (PC) 2 separated the samples clearly by line and accounted for 8.6% of the variance seen in the Harderian gland transcriptome (Figure 4-2). PC1 accounted for a large portion of the variance (51.1%), however, samples did not clearly cluster based on any known parameters. PC1 may be related to dpi because some time points clustered very closely on PC1 (Figure 4-2).

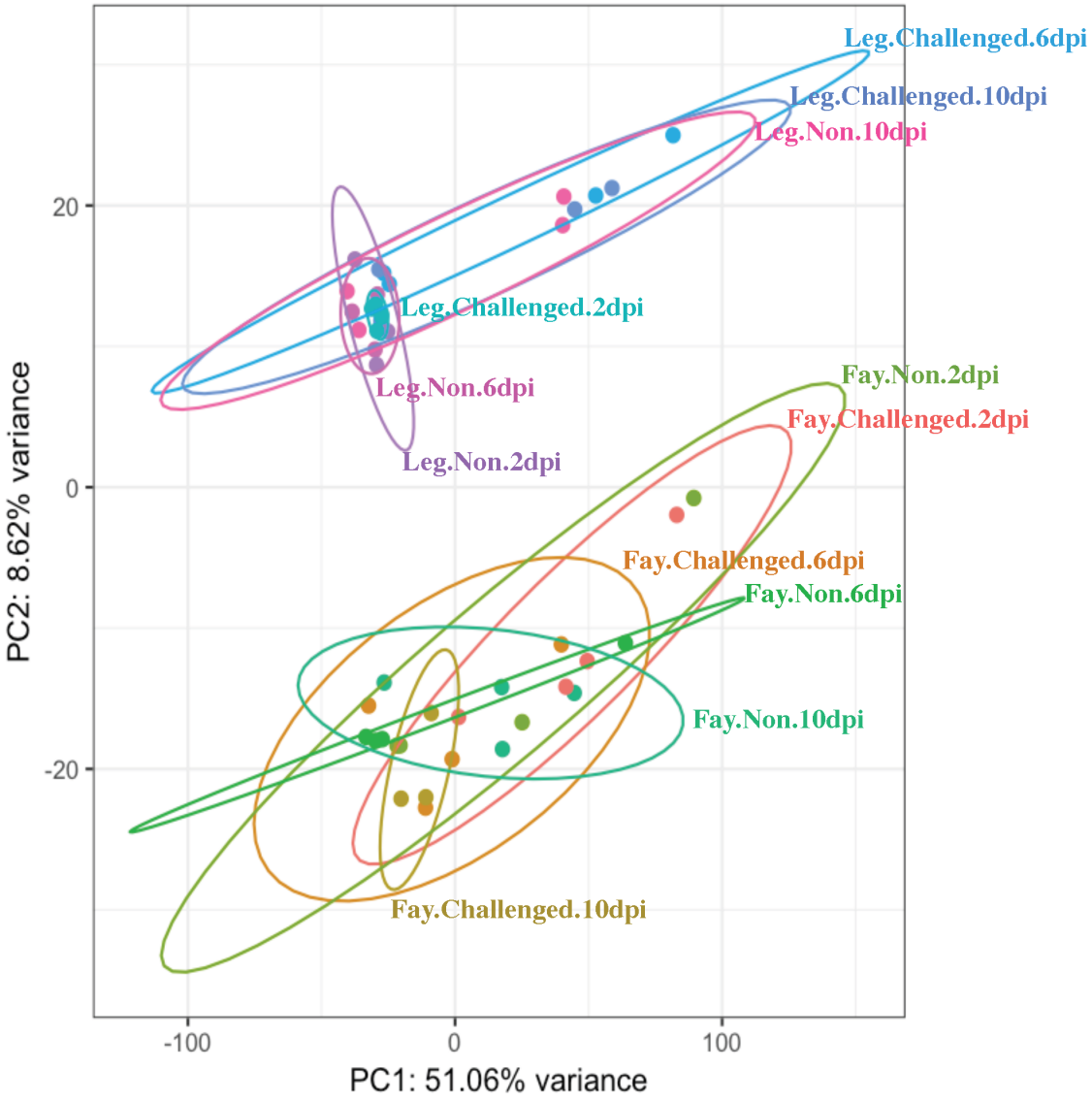

Figure 4-2: Principal component analysis plot generated by pcaExplorer. Each dot represents a sample colored to separate by line (Fayoumi: Fay; Leghorn: Leg), challenge status (Challenged; Nonchallenged: NON), and time (2, 6, and 10 dpi). Ellipses were drawn around each treatment group with 95% confidence. The top 5000 genes were used to calculate the principal components. Principal component 1 (PC1) accounts for 51.06% of the variance in the Harderian gland transcriptome. PC2 accounts for 8.62% of the variance and clusters clearly separate by line.

Challenged vs. nonchallenged birds by line and time

The challenge resulted in few DEG at most time points within each line (Table 4-2). Of the few DEG, several have known immune function (Table 4-2). The Leghorns at 6 dpi had much larger numbers of DEG (Table 4-2), which may be related to lack of viral

clearance at 6 dpi in the Leghorns as shown by higher viral transcript cpm values (Figure 4-1). Pathway analysis was performed to further characterize the response to challenge in the Leghorns at 6 dpi because of the large number of DEG (Table 4-2).

Table 4-2: Challenged vs. nonchallenged down and upregulated genes within each line and day

Line	DPI[a]	Downregulated[b]	Upregulated[c]	Immune Related Genes
Fayoumi	2	1	9	TNFRSF6B[c], IFI6[c], ZNFX1[c], Mx1[c]
Leghorn	2	2	17	C1S[b], MPEG1[c], TLR3[c], IFI6[c], ZNFX1[c], Mx1[c]
Fayoumi	6	0	0	---
Leghorn	6	57	623	C1S[c], ICOS[c], STAT1[c], CCR7[c], LITAF[c], ITK[c]
Fayoumi	10	5	18	IGFBP2[c], DNAJB11[b]
Leghorn	10	0	0	---

[a]Days Post Infection; [b]downregulated due to challenge FDR<0.05; [c]upregulated due to challenge FDR<0.05; TNF Receptor Superfamily Member 6b (TNFRSF6B); Interferon Alpha Inducible Protein 6 (IFI6); Zinc Finger NFX1-Type Containing 1 (ZNFX1); MX Dynamin Like GTPase 1 (Mx1); Complement C1s (C1S); Macrophage Expressed 1 (MPEG1); Toll-like receptor 3 (TLR3); Inducible T-Cell Costimulator (ICOS); Signal transducer and activator of transcription 1 (STAT1); C-C chemokine receptor type 7 (CCR7); Lipopolysaccharide Induced TNF Factor (LITAF); IL2 Inducible T-Cell Kinase (ITK); Insulin Like Growth Factor Binding Protein 2 (IGFBP2); DnaJ Heat Shock Protein Family (Hsp40) Member B11 (DNAJB11)

The top five canonical pathways with a z-score > 0.01 generated by IPA included the Th2 Pathway, Th1 Pathway, iCOS-iCOSL Signaling in T Helper Cells, CD28 Signaling in T Helper Cells, and PKCθ Signaling in T lymphocytes. All of these pathways were predicted to be activated and involved the T cell response. The B Cell Development Pathway was also significant and predicted to be activated, but was not among the top 5 canonical pathways. The upstream regulator T cell receptor (TCR) was predicted to be activated due to the increased expression of its target genes (Figure 4-3). The expression levels of these genes were also predicted to activate quantity of B lymphocytes, formation of lymphoid tissue, and cell proliferation of T lymphocytes and inhibition of replication of RNA virus (Figure 4-3). Thus, in response to NDV infection, at 6 dpi, the Leghorns were activating both the cell mediated and humoral arms of their adaptive immune response in this unique tissue.

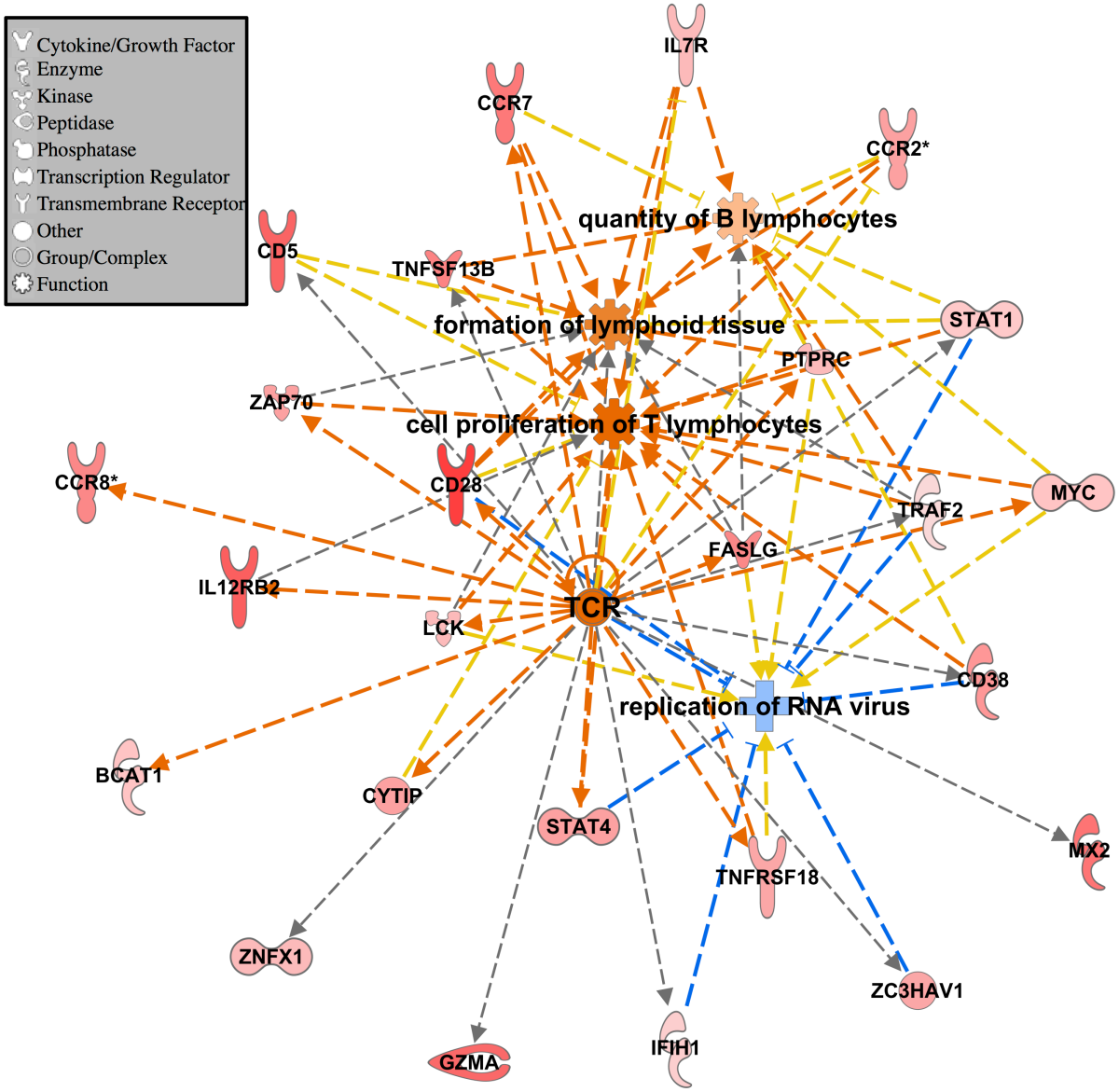

Figure 4-3: IPA analysis for the challenged vs. nonchallenged DEG in the Leghorn at 6 dpi. All genes input into IPA had FDR < 0.05 and absolute LFC >1. The larger the positive LFC for each gene, the darker the red fill. Genes and functions are connected with dashed lines that represent predicted activation (orange), an unknown relationship (grey), or a relationship that does not agree with the database (yellow). Orange fill represents a predicted activation, and blue, predicted inhibition. Genes in figure were grouped by the upstream regulator TCR, diseases and functions were added using the grow function in IPA ($p<0.0001$). Gene shapes represent their function.

Genetic line differences were apparent in the Harderian gland transcriptome in both challenged and nonchallenged birds at all times

The number of DEG at 2 dpi between the challenged Fayoumis and Leghorns was larger than between the age-matched, nonchallenged Fayoumis and Leghorns (Table 4-3).

This suggested that their transcriptomes became more different after challenge with the virus, even though within-line contrasts of challenged vs. nonchallenged birds showed few DEG at this time (Table 4-2). The extremely tight clustering of transcriptomes of the challenged Leghorns at 2 dpi (Figure 4-2) may have increased the ability to call genes as differentially expressed between the challenged Fayoumis and Leghorns. Overall, 58 genes were consistently differentially expressed in the Fayoumis and Leghorns at all times and in both challenged and nonchallenged birds. These DEG may be relevant to breed characterization. Somatotropin, for example, was always more highly expressed in the Fayoumis (Table 4-3) and has previously been associated with viral resistance to Marek's disease in chickens [20].

Table 4-3: Fayoumi vs. Leghorn differentially expressed genes within each challenge group and time point

		More Highly Expressed In		
Challenge Status	DPI[a]	Leghorn[b]	Fayoumi[c]	Immune Related Genes
Nonchallenged	2	181	196	TRAP1[b], Somatotropin[c], MZB1[c], IFNG[c]
Nonchallenged	6	144	263	CFAP221[b], Somatotropin[c], JCHAIN[c], CCR10[c]
Nonchallenged	10	87	87	Somatotropin[c], DPT[c]
Challenged	2	817	1481	TRAP1[b], MZB1[c], JCHAIN[c], CCR10[c]
Challenged	6	163	201	NFKBIZ[b], IL18[b], ZNF830[c]
Challenged	10	88	194	NFKBIZ[b], TRAP1[b], ACKR[c], TNFAIP6[c]

[a]Days Post Infection; [b]more highly expressed in the Leghorns FDR<0.05; [c]more highly expressed in the Fayoumis FDR<0.05; TNF receptor associated protein 1 (TRAP1); marginal zone B and B1 cell specific protein (MZB1); interferon gamma (IFNG); cilia and flagella associated protein 221 (CFAP221); joining chain of multimeric IgA and IgM (JCHAIN); C-C motif chemokine receptor 10 (CCR10); dermatopontin (DPT); NFKB inhibitor zeta (NFKBIZ); interleukin 18 (IL18); zinc finger protein 830 (ZNF830); atypical chemokine receptor 2 (ACKR); TNF alpha induced protein 6 (TNFAIP6)

Further analysis of the DEG between the Fayoumi and Leghorn revealed which pathways were more highly activated within each line (Figure 4-4). Many inflammatory pathways were more activated in the challenged Fayoumis at 2 dpi, and this may be related to the amount of viral transcript counts present at that time. Interestingly, most of the pathways that were differentially activated between the two lines appear to be immune related (Figure 4-4). Pathways in which the predicted activation was of different direction in challenged and nonchallenged birds are of particular interest.

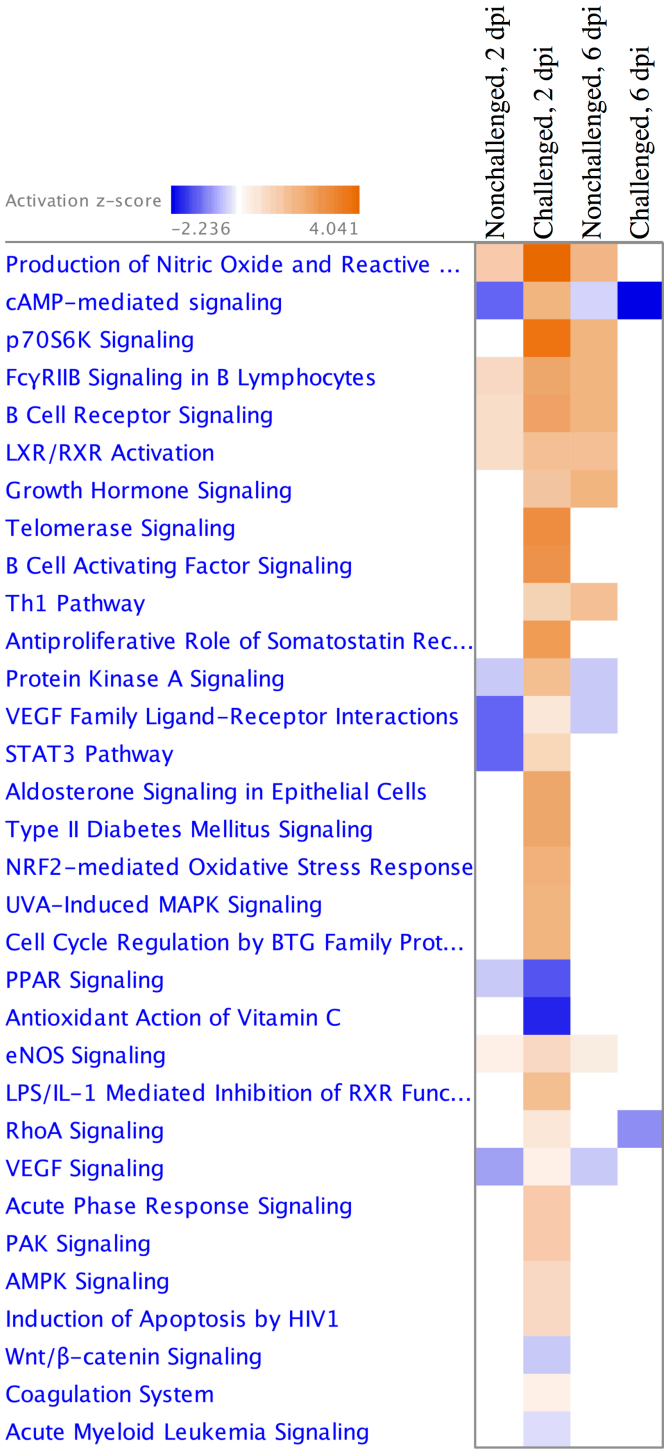

Figure 4-4: Pathways more differentially activated between the lines within each treatment group at 2 and 6 days post infection. The Fayoumis and Leghorns were directly compared within each dpi and challenge group. The resulting differentially expressed genes with FDR<0.10 were used as input into IPA for pathway analysis. Contrasts from 10 dpi were removed because no pathways were significant. Heat map shows pathways predicted to be relatively more activated in the Fayoumi (orange) and Leghorn (blue). The more intense a square in the heat map, the higher the z-score and prediction confidence. White squares represent contrasts with no or too few DEG within that pathway to make a prediction (z-score=0).

Genes impacted by the challenge*line interaction are important

Genes that were significantly impacted by the challenge*line interaction may offer insights into mechanisms of disease resistance or vaccine readiness. At 2 dpi, genes significantly impacted by the interaction included: Down Syndrome Cell Adhesion Molecule (DSCAM), Glutamate Ionotropic Receptor NMDA Type Subunit 1 (GRIN1), Synaptosomal-associated protein 25 (SNAP25), and CaM Kinase Like Vesicle Associated (CAMKV). At 6 dpi, no genes were significantly impacted by the challenge*line interaction. Phenylalanyl-TRNA Synthetase Alpha Subunit (FARSA), Carboxypeptidase A6 (CPA6), Ribosomal Protein Lateral Stalk Subunit P2 (RPLP2), and ENSGALT00000073955 were identified as significantly impacted by the challenge*line interaction at 10 dpi.

RNA-seq results were validated by high throughput qPCR

Fluidigm Biomark was used as a method of high throughput qPCR to validate the RNA-seq methods by correlating the log fold change (LFC) calculated by both techniques. Primers were previously published [17]. The six challenged vs. nonchallenged contrasts (within each time and line), were used to compare the RNA-seq LFC with the Biomark LFC calculated via the $-2^{-\Delta\Delta CT}$ method (Figure 4-5). The correlation (r=0.77) was strong.

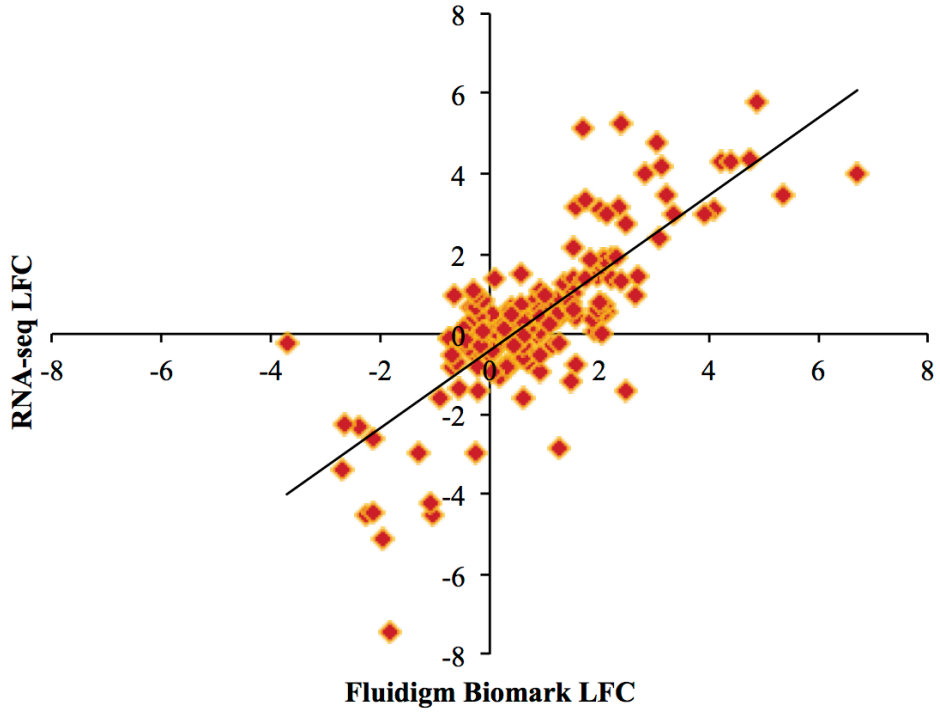

Figure 4-5: Scatterplot correlation between LFC estimated by RNA-seq and Fluidigm Biomark. Contrasts between challenged and nonchallenged birds within each line and time were used to generate this plot. Each point represents a gene's log fold change (LFC) as measured by RNA-seq (y-axis) and Fluidigm Biomark (x-axis. A total of 32 genes were analyzed across six different contrasts resulting in 192 data points. The correlation between the two technologies was r=0.77.

Discussion

There was a large number of DEG between the challenged Fayoumis and Leghorns at 2 dpi. This was surprising, because there were few DEG in the contrasts of challenged and nonchallenged birds within-line at 2 dpi. The PCA can explain some of this result. The transcriptome of challenged Leghorns at 2 dpi clustered very tightly together with the two largest principal components (which together account for 60% of the variance), much tighter than the nonchallenged Leghorns at 2 dpi. The challenged Fayoumis clustered more tightly than the nonchallenged Fayoumis as well at 2 dpi. A multi-dimensional-scaling plot showed the same clustering patterns as the PCA plot (data not shown). Less within group variation allows for the identification of more DEG. The PCA plot also showed the tight clustering of

the nonchallenged Leghorns at 6 dpi, which may have allowed for the detection of more DEG when contrasting the challenged and nonchallenged birds at this time. The samples within Leghorn treatment groups tended to diverge with time, whereas in the Fayoumis, the samples clustered more tightly as time progressed across PC1. It is unclear what drives some treatment groups to cluster more tightly than others; PC1 accounts for a large portion of variance and cannot be identified from the study parameters.

Previous studies have shown the influx of plasma cells and T cells to the Harderian gland after infection with NDV [5,11,12]. In the Harderian gland, plasma cells have been shown to synthesize mainly IgA [5] , which has been detected as early as 4 dpi in the saliva of Leghorns [21] after infection with NDV. However, no IgA was detected in the lachrymal fluid of the challenged Fayoumis or Leghorns at 2 & 6 dpi (data not shown).

The large number of DEG seen between the challenged and nonchallenged Leghorns at 6 dpi showed activation of the adaptive immune response in these birds. These DEG predicted the activation of several T cell related pathways. The activation of T cell related pathways after infection was also seen in the tracheal epithelial cells of these birds [17], but not in the lung, which had no detectable viral transcript counts [18]. T cell related pathways appear to be activated at sites of viral replication, which is likely a result of an increased T cell population. After infection with Hitchner B1 NDV strain, all T cell subsets increased at least 2-fold in the Harderian gland, and CD8+ T cells increased 6-fold [12].

There might be a relationship between the large number of DEG and the viral transcript counts in the Harderian gland of the Leghorns at 6 dpi. Viral persistence in the Leghorns could cause the large DEG response at 6 dpi in the Leghorns that was absent in the Fayoumis, because the Fayoumis cleared the virus by 6 dpi in the Harderian gland. It is

unknown, however, if the viral transcript counts correlate with the number of infectious viral particles. Surprisingly, the resistant Fayoumis had significantly more detectable viral transcript counts, as estimated from the RNA-seq, at 2 dpi than the susceptible Leghorns in the Harderian gland. This result should be explored further to determine whether a greater viral transcript counts is advantageous for mounting an appropriate immune response in the Harderian gland. Overall, the cpm for each viral gene detected in the Harderian gland was lower than that detected in the tracheal epithelial cells of these birds [17]. The different cell composition of these tissues may be one of the causes. Likely, a higher percentage of cells were susceptible to lentogenic NDV replication in the tracheal epithelial cells than the Harderian gland, as lentogenic NDV requires a protease found in epithelial cells to cleave the Fusion protein [22]. Also, unlike the trachea, in the Harderian gland the viral gene cpm did not decrease from 3' to 5' as would be expected [17]. The high cpm of the F and HN genes in the Harderian gland of challenged Fayoumi at 2 dpi may have facilitated the production of more neutralizing antibodies to these external structural proteins.

The two genetic lines clearly responded differently to the virus in the number of DEG and in the amount of detectable viral transcript counts. When the lines were directly compared, several immune related pathways differed in their relative activation. Relative to the Leghorns, the Fayoumis had more immune-related pathways that were in an activated state under resting conditions (nonchallenged), including eNOS signaling, Th1 pathway, LXR/RXR Activation, B cell receptor signaling, FcγRIIB signaling in B lymphocytes, and production of nitric oxide and reactive oxygen species. These pathways represent each facet of the immune system: innate, cell mediated, and humoral immunity. The relative activation of these immune pathways in the nonchallenged Fayoumis may be a reason for the low

numbers of DEG found between the challenged and nonchallenged Fayoumis at the time points measured. Differences in immune related pathways of nonchallenged Fayoumis and Leghorns was also observed in the spleen [19]. It is unknown whether the reason for the Fayoumi's resistant or Leghorn's susceptible phenotype is due to their response to pathogen or their constituitive expression levels. The increased relative activation of immune related pathways under homeostatic conditions may be partially responsible for the favorable phenotype observed in the Fayoumis. The Fayoumis may be more equipped to handle a viral infection under resting conditions and, therefore, do not need to mount a large immune response to clear the virus, as assessed by changes in gene transcription.

The genes that were significantly impacted by the challenge*line interaction are of particular interest. Of those genes, some, including RPLP2, DSCAM, and CPA6, have been shown to have immune function. Flaviviruses require RPLP2 for infection, because it is necessary for viral translation [23]. In arthropods, DSCAM plays a crucial role in the innate immunity's specificity [24]. In initial cancer stages, CPA6 mRNA is more highly expressed than in late stages [25]. These genes are of importance to those looking for mechanisms of disease resistance or the genetic impact on vaccination efficacy.

Conclusions

To the authors' knowledge, this is the first time the Harderian gland transcriptome has been analyzed using RNA-seq. The Harderian gland of two genetic lines showed very different responses to NDV in number of DEG and amount of detectable NDV. The pathways activated in the nonchallenged Fayoumi suggest a more elevated immune system relative to the Leghorn under resting conditions. The challenged Fayoumi had significantly higher viral transcript counts in the Harderian gland at 2 dpi but no longer had detectable

viral transcript counts at 6 dpi, unlike the Leghorn. This suggested the Fayoumis might clear the virus more quickly than the Leghorns. The Harderian gland is a first responder tissue after eye-drop vaccination or aerosol transmission of the virus. This study established a foundation for future research to investigate this tissue's unique role in host defense.

Methods

Animal experiment

This study was approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC log number 1-13-7490-G) and has been described previously [17,18]. All experiments were performed in accordance with the relevant guidelines and regulations. Two inbred lines, the Fayoumi (M 15.2) and Leghorn (GHs 6), from the Iowa State University Poultry Farm (Ames, IA), were used as a discovery platform to model relative resistance and susceptibility to NDV. Fayoumi and Leghorn breeders are a National Poultry Improvement Plan (NPIP) certified flock, tested and confirmed Salmonella and avian influenza virus free. Breeders of both lines are housed in the same building at the Iowa State University Poultry Farm (Ames, IA). At hatch, birds were placed in a biosafety level 2 facility. Three weeks later, the challenged birds (n=49) were inoculated with 200 μL of $10^7 EID_{50}$ of La Sota NDV via the ocular/nasal route, and the nonchallenged birds (n=40) were inoculated with PBS in the same manner. Challenged and nonchallenged birds were kept in separate rooms from hatch until the end of the experiment. Approximately, one third of the chicks were euthanized at 2, 6, and 10 dpi for tissue collection, and the Harderian gland was removed, placed into RNAlater (ThermoFisher Scientific, Waltham, MA) for approximately one week, removed from the RNAlater, and stored at -80°C. Each treatment group included four biological replicates for transcription analysis with the exception the Leghorns at 2 dpi (5 challenged, 3 nonchallenged) and 3 challenged Fayoumis at 10 dpi.

Total RNA was isolated from these samples using the RNAqueous kit (Thermo Fisher Scientific, Waltham, MA) and was DNase treated using a DNA-free kit (Thermo Fisher Scientific, Waltham, MA). All samples had an RNA quality number>8.0, as measured using the Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies, Inc., Ankeny, IA). The isolated RNA from each sample was used to independently construct two cDNA libraries for each biological replicate using the high-throughput protocol in the TruSeq RNA sample preparation guide (v2; Illumina, San Diego, CA). Libraries were validated using the Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies, Inc., Ankeny, IA) and 100 bp single end reads were sequenced on the HiSeq2500 (Illumina, San Diego, CA) at Iowa State University DNA Facility (Ames, IA). The sequencing data are available in the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6038.

RNA-seq pipeline

The sequence data were taken through a standard pipeline for analysis using FASTX, TopHat2 [26], and HTSeq [27] as described previously [18]. A chi-square test was performed on the HTSeq output for each pair of technical replicates to confirm the technical replicates had minimal differences. Then the raw counts were combined for each pair of technical replicates. One technical replicate was discarded because only 10% of its reads mapped to the chicken reference genome, indicating technical failure.

Reads that did not map to the chicken reference genome were analyzed further as described previously [17]. These unmapped reads from each technical replicate were mapped to the La Sota genome (GenBank accession number JF950510.1) using BWA [28] and the mapped reads were counted with HTSeq [27]. The technical replicates' raw counts for each viral gene

were summed for each biological replicate. The counts per million for each viral gene for each biological replicate were statistically analyzed in JMP statistical software (JMP Group Inc., San Francisco, CA) using a standard least squares, effect leverage full factorial test that accounted for line, dpi, gene, and their interactions.

Principal component analysis

For data visualization, pcaExplorer [29] was utilized. The dds function from DESeq2 [30] was used for normalization, accounting for line, challenged, dpi, and sex in the model, and the rlogtransformation in pcaExplorer was used. The 5000 transcripts that contributed the most variance were used to calculate the variance associated with the principal components.

Differential expression analysis

For differential expression analysis of the count data, the generalized linear model in edgeR [31] accounted for line, challenge, and dpi to determine the number of DEG (False Discovery Rate (FDR) <0.05). Contrasts were written to compare challenged and nonchallenged birds within each line and time, Fayoumis and Leghorns within each challenge group and time, and the challenge*line interaction at each time.

The edgeR output, including LFC and FDR for each transcript for each contrast, was input into Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA). The results from the challenged vs. nonchallenged Leghorns at 6 dpi contrast were input into IPA, transcripts with FDR<0.05 and absolute LFC > 1 were used for an expression analysis. Figure 4-3 was generated based on the upstream regulator TCR (activation z-score = 2.712; p-value = 7.52E-10) from the upstream analysis portion in IPA. Additional diseases & functions (p-value <0.0001) relevant to NDV challenge were added to the network using the grow function (Figure 4-3). The results from the Fayoumi vs. Leghorn contrasts were input into IPA and

transcripts with FDR <0.10 were used for a comparison analysis for both challenged and nonchallenged birds at 2 and 6 dpi (Figure 4-4); no pathways were significantly different between the two lines at 10 dpi. IPA calculates a z-score based on the expression levels of genes within a pathway or related to a functional term. If a z-score is positive IPA predicts activation of a pathway or function, and if negative IPA predicts inhibition of a pathway or function.

Method validation

To validate the RNA-seq methodology, high-throughput qPCR using the Fluidigm Biomark system was performed. The methods [17,18] and primers [17] were reported previously. The LFC calculated by edgeR for the RNA-seq data and the $-2^{-\Delta\Delta CT}$ method from the Fluidigm Biomark output was used for the correlation of the challenged vs. nonchallenged comparisons at each time and within each line for 32 genes, resulting in 192 data points.

Author contributions

MSD: Collected samples, isolated RNA, constructed cDNA libraries, processed and analyzed RNA-seq data, wrote paper
RAG: Experimental design, prepared viral isolate for inoculation, reviewed and edited paper
DAB: Experimental design, reviewed and edited paper
TRK: Reviewed and edited paper
JCMD: Experimental design, advised on statistical analyses, reviewed and edited paper
HZ: Experimental design, reviewed and edited paper
SJL: Experimental design, reviewed and edited paper, oversaw analysis of data

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CHAPTER 5. TISSUE TRANSCRIPTOME JOINT ANALYSIS AFTER VACCINE CHALLENGE IN INBRED CHICKEN LINES OF DIFFERENTIAL RESISTANCE TO VIRULENT NEWCASTLE DISEASE VIRUS

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Abstract

The decrease in production and the mortality caused by infections with virulent Newcastle disease virus (NDV) have devastating impacts on poultry, especially in developing countries. The current study utilized two inbred chicken lines, Fayoumi and Leghorn, which have been reported to be relatively more resistant and susceptible, respectively, to NDV. Previous studies demonstrated differential response of these lines to infection with the La Sota NDV vaccine strain. To further determine whether these lines also differ in response to virulent NDV field strains, birds from both breeds were challenged with various doses of a virulent NDV strain that was isolated from a chicken in Burkina Faso in 2008. At high challenge doses, the Fayoumis had significantly longer survival times than the Leghorns, further validating the biological model of relative resistance and susceptibility to NDV of these two genetic lines.

To gain more insight into the response to NDV at the whole-organism level, transcriptome data of multiple tissues (lung, trachea and Harderian gland) were jointly

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analyzed. These data were generated from lentogenic NDV-challenged birds and had previously been analyzed individually for each tissue. The current study utilized weighted gene co-expression network analysis (WGCNA) to identify modules of co-expressed genes that associated with traits of interest. Three modules were associated with genetic line or challenge status, and further analyzed with functional gene ontology (GO) term and network analysis. We identified driver genes with high gene significance and module membership, such as *EIF2AK2*, *MPEG1*, and *TNFSF13B*. These genes involved in both the innate and adaptive immune response were likely critical in host defense to the virus. This study provided new insights into the host response to the NDV vaccine strain and better established the Fayoumis and Leghorns as genetic resources to study resistance to virulent NDV strains.

Introduction

Newcastle disease virus (NDV) is a single-stranded, negative sense, RNA virus. The clinical signs associated with NDV infection in chickens range from subclinical to 100% mortality depending on the virulence of the infection strain (1). NDV is classified into five pathotypes based on clinical signs: asymptomatic enteric, lentogenic, mesogenic, velogenic viscerotropic, and velogenic neurotropic (2). Velogenic strains, also known in the United States as exotic NDV (END virus), have the most detrimental impacts on the poultry industry. Infection with velogenic neurotropic NDV can result in neurological clinical signs, whereas velogenic viscerotropic strains cause intestinal lesions (2). Both velogenic strains result in high morbidity and mortality (2). The lentogenic strains are the least virulent and because all NDV strains belong to the same serotype, lentogenic strains are used for vaccination (3). Lentogenic NDV is limited in its replication to host cells that produce a trypsin-like protease to cleave the fusion protein (4). This restricts lentogenic NDV

replication to epithelial cells where this protease is expressed (5). Hence, lentogenic strains have the largest impact near sites of infection such as the trachea, lung, and Harderian gland, all of which contain epithelial cells and are also involved in mucosal immunity. These tissues, in which the virus and host have direct contact, are ideal for examining host-pathogen interaction. Increased understanding of the host-pathogen interaction creates the potential for improving strategies to curb the negative impacts of NDV.

Two inbred chicken lines have been shown to differ in their susceptibility to lentogenic La Sota NDV (6), but it is unknown if the relative resistance of the Fayoumis (M 15.2) compared to the Leghorns (GHs 6) is also the case when challenged with a velogenic NDV strain or isolate. These lines have also shown varying responses to avian influenza (7), Marek's disease (8), *Salmonella* (9), and *Eimeria* (10). Inbred lines that differ in their relative susceptibility offer an excellent tool to study mechanisms of disease resistance.

The RNA-seq approach shows the genes and pathways impacted by a treatment; however, it only shows a snapshot of what is occurring in a particular tissue and time. Because the transcriptome is not stagnant, a more comprehensive physiological picture can be obtained by an integrative analysis of the transcriptomes of multiple tissues at multiple time points after a treatment. Previously, the tracheal epithelial cells, lung, and Harderian gland of Fayoumis and Leghorns at 2, 6, and 10 days post infection (dpi) with lentogenic NDV have been individually analyzed with RNA-seq (6, 11, 12). The three tissues had very distinct responses to NDV (6, 11, 12). Taking a systems biology approach and analyzing the three tissues together will provide a more comprehensive picture of how the whole animals responded to NDV. Prior joint-tissue analyses using microarray or RNA-seq to study the impact of avian pathogenic *E. coli* on broiler chickens have been performed (13, 14).

Improvements in the technologies and tools for analyzing this type of data have enabled a more thorough examination of these complex data.

The objectives of this study were to gain a more comprehensive understanding of the chicken's response to NDV at the transcriptome level and identify genes potentially associated with resistance to lentogenic NDV. Additionally, the relative resistance of Fayoumis versus Leghorns to velogenic NDV was assessed.

Results

Confirmation of the Fayoumi's relative resistance to NDV after challenge with a velogenic strain

All birds were negative for NDV antibodies prior to challenge. After challenge with velogenic NDV, the Fayoumis survived longer than the Leghorns at all inoculation doses (Figure 5-1). As expected, the average survival time decreased as the inoculation dose increased. All infected birds succumbed to velogenic NDV by 13 dpi. The difference between the Fayoumi and Leghorn average survival time increased as inoculation dose increased (Figure 5-1). Proportional Hazards test and parametric survival analysis both showed that the main effects of line and dose each had a significant impact ($p<0.001$) on the survival time. A log-rank test showed a significant difference between the average survival time of the Fayoumis and Leghorns at 10^4 ($p=0.0253$) and 10^5 ($p=0.0085$) mean embryo infectious dose (EID_{50}) per 100 µL.

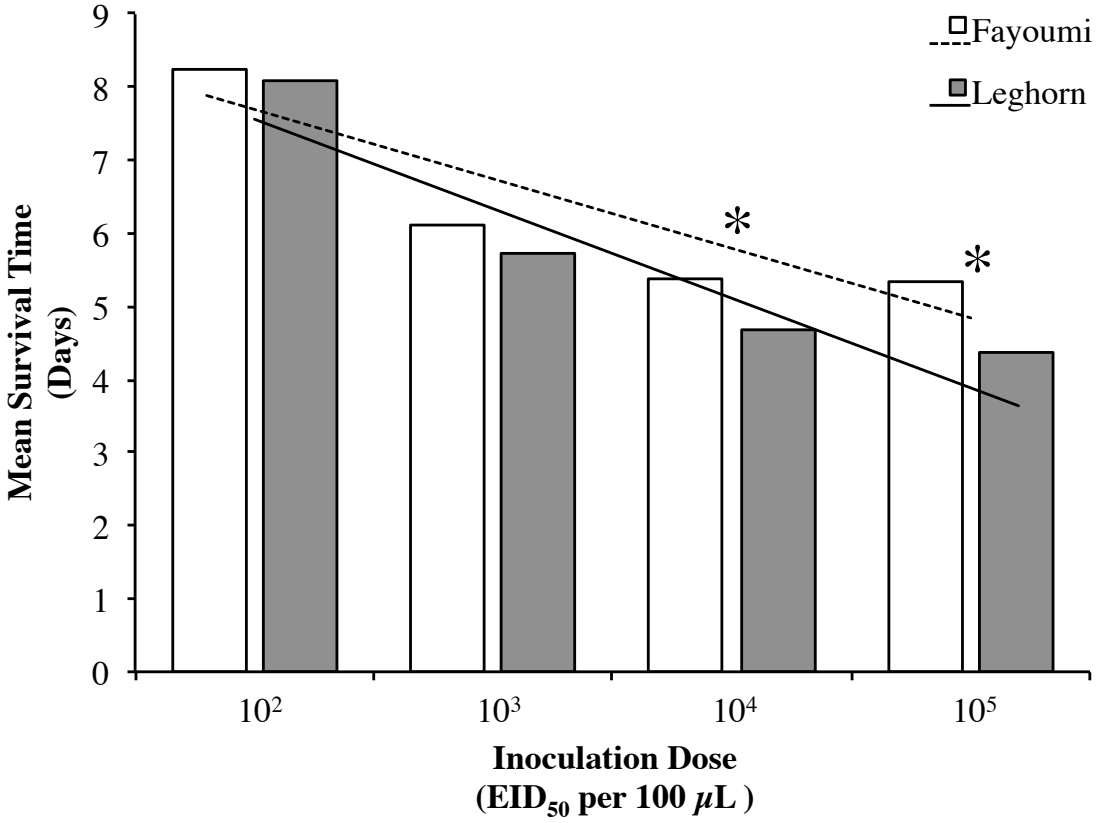

Figure 5-1: Average survival time of the Fayoumis and Leghorns at different inoculation doses of velogenic NDV. Average survival time (days; y-axis) and inoculation dose (EID_{50} per 100 μL; x-axis). The regression of mean survival time on the inoculation dose is shown for the Fayoumi (dashed) and Leghorn (solid), calculated in Microsoft Excel. Asterisk (*) indicates inoculation doses for which there was a significant difference ($p<0.05$) between the mean survival time in the Fayoumis and Leghorns.

Principal component analysis of the trachea, lung, and Harderian gland

The transcriptomes of three tissues from birds infected with lentogenic NDV were jointly analyzed to more comprehensively characterize the host response to NDV. Principal component analysis showed a clear separation between the tissues, as expected (Figure 5-2). Principal component (PC) 1 (PC1 = 51.91%) separated all three tissues and PC2 (32.11%) separated the trachea and Harderian gland from the lung (Figure 5-2). No clear clustering was observed by any other parameter in all tissues.

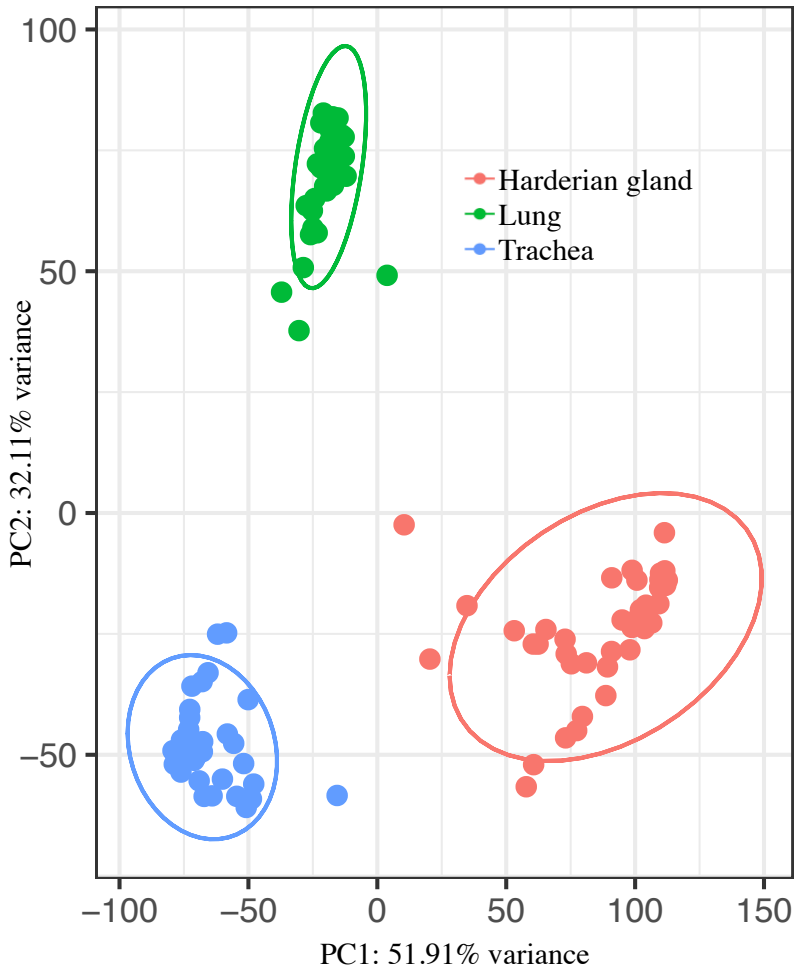

Figure 5-2: Principal component analysis shows clear separation by tissue. pcaExplorer generated PCA plot using top 1000 most variant transcripts. Ellipses were drawn around groups with 95% confidence interval. Groups separated by tissue, lung (green), trachea (blue), Harderian gland (pink).

Differential expression analysis

A differential expression analysis was performed to determine if transcripts responded differently to the lentogenic NDV challenge between tissues. The numbers of differentially expressed genes (DEG) for the challenge by tissue interaction, within each line and days post infection (dpi), are shown in Table 5-1. Large numbers of genes with differential response to infection between the tissues were identified by the interaction between the lung and Harderian gland responses in the Fayoumis at 10 dpi (Table 5-1) and separately the large numbers of DEG in the Leghorns at 6 dpi (Table 5-1) may correspond to

large numbers of DEG due to challenge within an individual tissue (11, 12). With the exception of the Fayoumis at 10 dpi and the Leghorns at 6 dpi, there were few DEG between the lung and Harderian gland. Interestingly, there were no DEG at 10 dpi in the Leghorns and few DEG in the Fayoumi at 6 dpi (Table 5-1). Overall, there were differences in the number of DEG from each interaction contrast, depending on genetic line and time. Of the 18 contrasts examined, *CD8B* and *LY6D* were differentially expressed most frequently in 7 and 6 contrasts, respectively.

Table 5-1: Comparing the tissues' response to lentogenic challenge within each line and time.

Line	Time	Interaction contrast [a]	DEG [b]
Fayoumi	2 dpi	Lung vs. Harderian gland	10
		Trachea vs. Harderian gland	151
		Trachea vs. Lung	135
	6 dpi	Lung vs. Harderian gland	0
		Trachea vs. Harderian gland	1
		Trachea vs. Lung	3
	10 dpi	Lung vs. Harderian gland	1,523
		Trachea vs. Harderian gland	54
		Trachea vs. Lung	20
Leghorn	2 dpi	Lung vs. Harderian gland	0
		Trachea vs. Harderian gland	141
		Trachea vs. Lung	115
	6 dpi	Lung vs. Harderian gland	743
		Trachea vs. Harderian gland	877
		Trachea vs. Lung	226
	10 dpi	Lung vs. Harderian gland	0
		Trachea vs. Harderian gland	0
		Trachea vs. Lung	0

[a] Within each line and dpi: (Tissue1, Challenged - Tissue1, Nonchallenged) - (Tissue2, Challenged – Tissue2, Nonchallenged); [b] Differentially Expressed Genes (FDR<0.05)

Gene co-expression analysis in the trachea, lung, and Harderian gland after lentogenic challenge

The module-trait relationship analysis resulted in several associations between the modules generated by WGCNA based on co-expression and factors of the study: line, dpi, challenge status, sex, tissue (Figure 5-3). The lightyellow module strongly positively

correlated with line (0.99 for Fayoumi=1 versus Leghorn=0, p=1e-120), but did not significantly correlate with any other factor, suggesting for the 161 transcripts within this module, there was no interaction between line and any other factor. The lightyellow module could help better characterize the inherent differences between these two inbred genetic lines. The orange module was strongly positively correlated with sex (0.97 for male=1 versus female=0, p=1e-90). Within this module, 17 transcripts have not been placed on a chromosome, 7 are on the W chromosome, and 94 on the Z chromosome. No correlations with other modules show these transcripts are only impacted by sex.

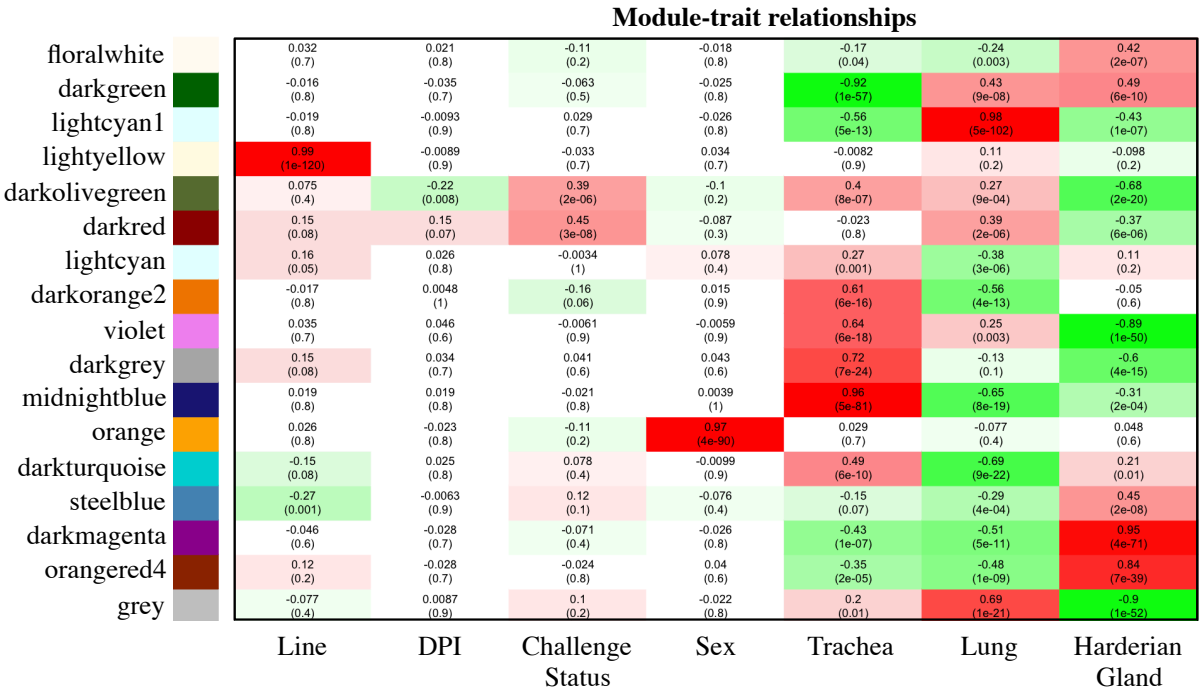

Figure 5-3: Module-trait relationships calculated by WGCNA. Factors of interest (x-axis) were correlated to each module (y-axis). The first value within each square is the correlation and the second value in parenthesis is the p-value of the association. The more positively correlated the module and the factor, the more red the square, the more negatively correlated, the more green. Coding for the factors was as follows: Line [Fayoumi (1), Leghorn (0)], days post infection [dpi; 2 dpi (0), 6 dpi (1), 10 dpi (2)], NDV [challenged (1), nonchallenged (0)], sex [male (1), female (0)], Trachea [Trachea (1), Lung (0), Harderian gland (0)], Lung [Trachea (0), Lung (1), Harderian gland (0)], and Harderian gland [Trachea (0), Lung (0), Harderian gland (1)].

Three modules, midnightblue, lightcyan1, and darkmagenta, may be related to the specific functions of each tissue. The 1,308 transcripts in the midnightblue module were strongly positively correlated and more highly expressed in the trachea (0.96 for trachea=1 versus lung and Harderian gland=0, p=5e-81). The 1,720 transcripts in the lightcyan1 module were strongly positively correlated with and more highly expressed the lung (0.98 for lung=1 versus trachea and Harderian gland=0, p=5e-102). The large darkmagenta module (3,154 transcripts) strongly positively correlated with and more highly expressed in the Harderian gland (0.95 for Harderian gland=1 versus trachea and lung=0, p=4e-71).

The darkolivegreen module was negatively correlated with dpi (-0.22 for 2 dpi=0, 6 dpi=1, versus 10 dpi=2, p=0.0008), positively correlated with challenge status (0.39 for challenged=1 versus nonchallenged=0, p=2e-06), and negatively correlated with the Harderian gland (-0.68 for Harderian gland=1 versus trachea and lung=0, p=2e-20). The 80 transcripts in this module were likely important for the chicken's early response to NDV, because the negative correlation with dpi suggests they were more highly expressed at early time points. The darkred module was positively correlated with challenge status (0.45 for challenged=1 versus nonchallenged=0, p=3e-08), positively correlated with the lung (0.39 for lung=1 versus trachea and Harderian gland=0, p=2e-0.6), and negatively correlated with the Harderian gland (-0.37 for Harderian gland=1 versus trachea and lung=0, p=6e-06). Although not significant, this module showed a weak correlation with line (0.15 for Fayoumi=1 versus Leghorn=0, p=0.08). The darkred module potentially included genes related to resistance to NDV because of its associations with challenge status and line. The 333 transcripts in the steelblue module negatively correlated with line (-0.27 for Fayoumi=1 versus Leghorn=0, p=0.001), and positively correlated with the Harderian gland (0.45 for

Harderian gland=1 versus trachea and lung=0, p=4e-71). Although not significant, this module weakly correlated with challenge status (0.12 for challenged=1 versus nonchallenged=0, p=0.1). The steelblue module may also include transcripts potentially associated with resistance to NDV.

Genes that are co-expressed are likely to be regulated by similar mechanisms. Within each module, the transcripts with high gene significance (GS) and module membership (MM), i.e. driver genes, were likely to have the most influence on the expression values of the entire module. The top driver genes were identified for the module that was most highly correlated with each factor (Table 5-2). Several lincRNAs and unnamed protein coding genes were identified as driver genes (Table 5-2). The *NKX2-1* was among the top ten bottom loading genes for PC1, and *CLDN18* among the top ten top loading genes for PC2 calculated by pcaExplorer (15).

Functional analysis of modules of interest for birds infected with lentogenic NDV

The darkolivegreen, darkred, and steelblue modules were of particular interest because of their correlations with either challenge status or line. The darkolivegreen module's correlations with dpi and challenge status suggest that these genes were important in the early response to NDV. Of the 80 transcripts, 59 had an associated gene name. No modules were significantly correlated with both the challenge status and line; however, the darkred and steelblue modules may include genes associated with resistance to NDV because of their correlations with challenge status or line. Gene ontology (GO) term and network analysis was performed on these three modules. Panther identified significant top-level GO terms for the darkolivegreen, darkred, and steelblue modules (Figure 5-4). The significant GO terms for the darkolivegreen and darkred modules, both significantly correlated with

challenge status, were clearly immune related. The significant GO terms for the steelblue module were more general in nature (Figure 5-4).

Table 5-2: Top three driver genes from the module most highly correlated with each factor for birds infected with lentogenic NDV

Factor / Module	Transcript ID / Gene Name	GS	MM
Line / lightyellow	ENSGALT00000082473 / lincRNA	0.97	0.96
	ENSGALT00000065772 / lincRNA	-0.97	-0.97
	ENSGALT00000087010 / protein coding	0.96	0.97
DPI / darkolivegreen	ENSGALT00000017183 / *EIF2AK2*	-0.37	0.83
	ENSGALT00000016499 / *CMTR1*	-0.37	0.74
	ENSGALT00000066953 / protein coding	-0.35	0.90
Challenge status / darkred	ENSGALT00000027228 / *TNFSF13B*	0.53	0.82
	ENSGALT00000044004 / *MPEG1*	0.52	0.82
	ENSGALT00000003281 / protein coding	0.51	0.74
Sex / orange	ENSGALT00000077789 / *Nipped-B homolog-like*	-0.98	-0.95
	ENSGALT00000047883 / protein coding	-0.98	-0.96
	ENSGALT00000080994 / protein coding	-0.97	-0.95
Trachea / midnightblue	ENSGALT00000021346 / *akr*	0.97	0.96
	ENSGALT00000049178 / *DNAL4*	0.97	0.95
	ENSGALT00000009017 / *F3*	0.97	0.94
Lung / lightcyan1	ENSGALT00000089244 / protein coding	0.99	0.98
	ENSGALT00000010644 / *CLDN18*	0.99	0.97
	ENSGALT00000057049 / lincRNA	0.98	0.97
Harderian gland / dark magenta	ENSGALT00000059647 / *LRRC30*	0.98	0.98
	ENSGALT00000006028 / *PABPC4*	0.97	0.94
	ENSGALT00000059800 / *NKX2-1*	-0.97	-0.94

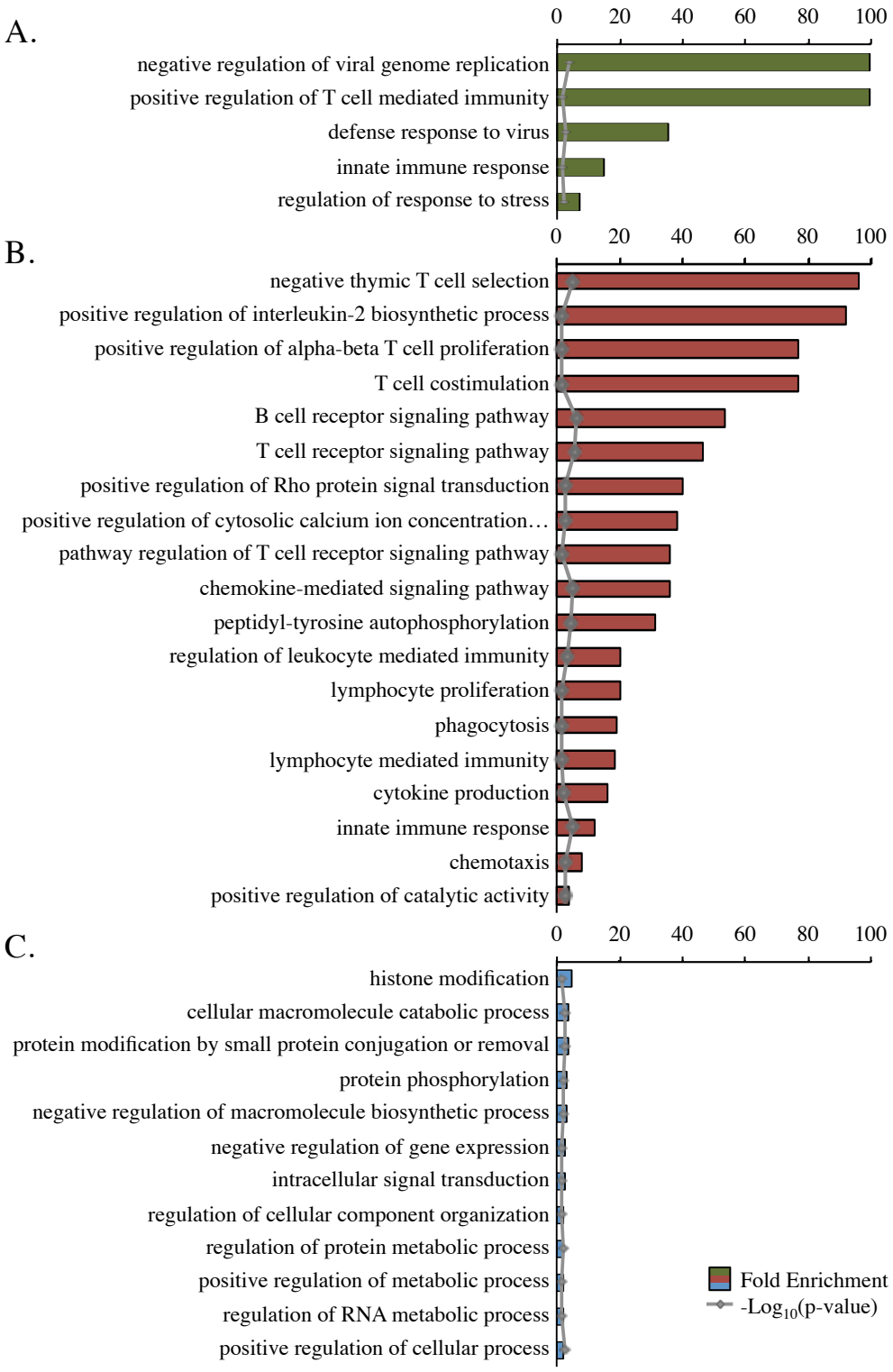

Figure 5-4: Fold enrichment of top-level overrepresented GO terms within modules of interest. Panther overrepresentation test using associated gene names for each of three modules, (A) darkolivegreen module, (B) darkred module, (C) steel blue module. Fold enrichment was capped at 100. $-Log_{10}$(p-value) shown in grey. All p-values<0.05. "…" = involved in phospholipase C-activating G-protein coupled signaling.

To generate protein interaction networks for the transcripts in each module of interest, STRING was utilized (Figure 5-5). The darkolivegreen, darkred, and steelblue networks had significantly more connections than would be expected by chance. The darkolivegreen module included 55 nodes and 31 edges and was significantly associated with several KEGG pathways that are related to response to virus, including Influenza A, Herpes simplex infection, and the RIG-I-like receptor signaling pathway (Figure 5-5A). Within the darkolivegreen module, *STAT1* had the second highest gene significance for challenge status and a high module membership (GS=0.50, MM=0.88), making the highly connected protein a potential driver gene. The darkred module network included 110 nodes and 60 edges and was significantly associated with the following KEGG pathways: Cytokine-cytokine receptor interaction, Cell adhesion molecules (CAMs), Phagosome, Jak-STAT signaling pathway, Intestinal immune network for IgA production, and Phosphatidylinositol signaling system. The fourth highest driver gene, *INPP5D* (GS=0.49, MM=0.84), was moderately well connected (Figure 5-5B). The network generated based on the steelblue module included 251 nodes and 78 edges and was significantly associated with only one KEGG pathway: Endocytosis. *DGKE* and *RICTOR* were among the top 4 driver genes in the steelblue module based on gene significance for line.

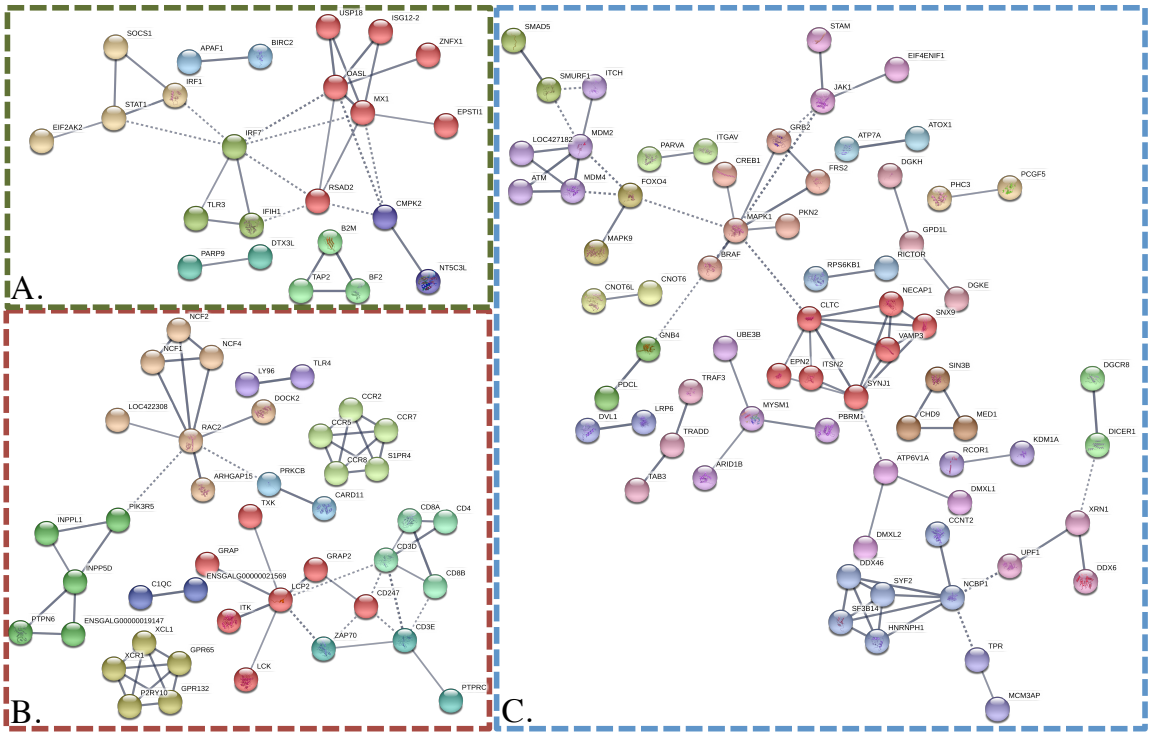

Figure 5-5: Network analysis of modules of interest. STRING was utilized to generate networks based on the associated gene name of the transcripts within the modules of interest: (A) darkolivegreen module, (B) darkred module, (C) steelblue module. Disconnected nodes were removed, high confidence (0.700), and MCL clustering (inflation=3).

Discussion

Overall, this study provided a comprehensive picture of how the Fayoumi and Leghorn responded to lentogenic NDV challenge in the transcriptomes of tissues where the NDV vaccine replicates. It also further confirmed the relative resistance and susceptibility of the Fayoumi and Leghorn, respectively, to NDV by demonstrating significant survival time differences after velogenic NDV infection. The Fayoumi is relatively more resistant to both lentogenic and velogenic NDV. This discovery platform enhances the ability of researchers to identify molecular and cellular mechanisms of disease resistance to NDV.

Prior analysis of each individual tissue's transcriptome resulted in unique findings. The tracheal epithelial cells had the highest amount of detectable virus (6). As time progressed, the number of DEG decreased between the challenged and nonchallenged birds

in the trachea, suggesting the trachea was consistently active in host response and in recovery from the virus in both lines (6). The PCA suggested the transcriptomes of the lung and Harderian gland were more impacted by line than by the challenge (11, 12). The Leghorn lung appeared to be non-responsive to the virus, whereas the Fayoumi lung produced a large number of DEG at 10 dpi when comparing the challenged to nonchallenged birds (11). The Harderian gland of the Leghorns showed a large number of DEG between the challenged and nonchallenged birds at 6 dpi, and the Fayoumis had very few DEG at all time points (12). The challenge by tissue interaction resulted in more DEG if there were more DEG between challenged and nonchallenged birds within a tissue. It is difficult to interpret the results of the challenge by tissue interaction because the tissues had time and line dependent responses to the virus as measured by DEG and had different amounts of detectable viral transcripts (6, 11, 12).

The complex design of the current combined, three-tissue experiment did not readily lend itself to a differential expression analysis because with three tissues, three time points, two genetic lines, and two challenge statuses, there was a very large number of possible contrasts. Using WGCNA for co-expression analysis does not rely on specific contrasts and can identify correlations between co-expressed genes and factors of importance in the study design. The darkolivegreen module was composed of genes that had a clear functional role in host defense. The unannotated transcripts within the darkolivegreen module should be explored further to identify their functions because they were co-expressed with genes important to the host response to NDV.

WGCNA was also used to analyze the lung tissue individually (11), which showed overlapping results with the joint tissue analysis. ENSGALT00000087010 was among the

top driver genes for line in the lung analysis as well as the joint analysis. In addition, *Nipped-B homolog-like* and ENSGALT00000080994 were among the top driver genes for sex in both analyses.

GO term analysis of the genes in the darkolivegreen module showed they were clearly involved in the host response to virus. *EIF2AK2* (also known as *PKR*) was the top driver gene for dpi for the darkolivegreen module. *EIF2AK2* is interferon inducible and has important antiviral effects (16). The EIF2 signaling pathway is known to inhibit NDV replication (17). This pathway was shown to be significantly different between the two lines in both the trachea (6) and spleen transcriptomes (18). *EIF2AK2* phosphorylates *EIF2α*, which regulates protein synthesis (19). Also, treatment with poly (I:C) led to expression changes of *EIF2AK2* in chickens (20). The negative correlation of the darkolivegreen module with dpi suggests *EIF2AK2* is likely more highly expressed at earlier time points post-infection Differential expression confirmed that the challenged birds had significantly higher *EIF2AK2* expression levels at 2 dpi when compared to 6 or 10 dpi across all tissues (data not shown). At 2 dpi, the highest levels of viral transcripts were detected in the trachea (6) and Harderian gland (12) as measured by RNA-seq; high expression of *EIF2AK2* at 2 dpi would aid in viral clearance.

The functions of the genes in the darkred module involve both the innate and adaptive immune system, as determined by the GO term analysis and the top two driver genes. It is well known that TNF genes play an important role in response to pathogens. *TNFSF13B* (also known as *BAFF*) specifically aids in survival and proliferation of chicken B cells (21), and was the top driver gene for challenge status in the darkred module. *TNFSF13B* receptors are expressed predominantly on chicken B cells (21). One receptor for *TNFSF13B*,

TNFRSF13B, was among the genes significant in the trachea challenge by line interaction at 6 dpi (6) and was differentially expressed between the Fayoumi and Leghorn chickens in the lung in both nonchallenged birds and birds challenged with avian influenza (7). NDV requires antibody production for viral clearance (22, 23) and expression of *TNFSF13B* may critically influence B cells and their antibody production in response to NDV.

Another driver gene for challenge status in the darkred module was *MPEG1*. *MPEG1* (also known as *Perforin-2*) was expressed highly in macrophages and is an important component of the innate immune system (24). The protein encoded by *MPEG1* works with complement to form membrane attack complexes and create holes in a target's cell membrane (24). *MPEG1* was 1 of 19 DEG in the Harderian gland (12) and 1 of 16 DEG in the lung (11) between challenged and nonchallenged Leghorns at 2 dpi.

The two lines have previously shown differences in the expression levels of *TNFSF13B* and *MPEG1* in response to viral pathogens, which may suggest a potential role in disease resistance. *MPEG1* and *TNFSF13B*, however, were not included in the darkred network. This suggests that these proteins may have unknown or low confidence connections in STRING to the other proteins in the darkred module, or that co-expression of these genes was due to their functional similarity and that their proteins may not interact.

The steelblue module significantly correlated with line and weakly correlated with challenge status. Although no immune related GO terms were significant, network analysis revealed immune related genes in this module, including *JAK*, *TRAF3*, *DICER1*, and *MAPK1*. This network was also associated with the Endocytosis pathway, which is a mechanism by which NDV enters the host cell. It may be possible for a host to modify gene expression of this pathway to minimize viral entry.

The current study's joint analysis of three target tissues generated a more comprehensive understanding of response to infection with lentogenic NDV at the whole organism level. The darkred and steelblue modules were the modules most likely to contain genes that relate to disease resistance, and *EIF2AK2*, *MPEG1*, and *TNFSF13B* are promising candidate genes for NDV resistance. This study is the first to report the difference in relative resistance of the Fayoumi and Leghorn chicken line to velogenic NDV, which validates this discovery platform for deeper understanding of the biological basis of resistance to both low and high pathogenic NDV strains.

Methods

Velogenic challenge and survival analysis

The experiments described in this study were conducted in accordance with protocols reviewed and approved by the Southeast Poultry Research Laboratory Institutional Animal Care and Use Committee (IACUC), and as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Fayoumi and Leghorn chicks were transported from Iowa State University (Ames, IA) to the Southeast Poultry Research Laboratory (Athens, GA) of the USDA to be challenged with velogenic NDV. In total, 175 10-day-old chickens, 66 Fayoumi and 109 White Leghorn chickens, were placed into brooding cages in ABSL-2 with 12 birds placed per cage with *ad libitum* feed and water. At three-weeks of age, the birds were moved to ABSL-3 and were randomly placed into plastic-molded isolators with 10 to 11 birds per isolator. Each of the 16 isolators had four to five Fayoumi and six to seven White Leghorn chickens. In ABSL-3, birds were checked daily to assess their health status, and they were kept at temperatures ranging from 23.3 to 25°C, with *ad libitum* access to food and water.

The day the birds were placed into ABSL-3, 200- 300 μl of blood was taken from each bird to confirm that all birds used in the study were negative for NDV antibodies. Blood samples were left to settle and coagulate and sera were decanted in new tubes. The sera samples were tested for presence of NDV antibodies using a hemagglutination-inhibition assay. Each bird was randomly placed into one of four virus challenge dosage groups: mean embryo infective dose (EID_{50}) of 10^5, 10^4, 10^3, or 10^2 per 100 μl, or a negative control group with diluent with no virus. Birds were challenged with a virulent NDV strain chicken/Burkina Faso/2415-580/2008 with 100 μl divided into both eyes and the choanal slit. The three higher viral doses each had four isolators each, and the lowest dose and the negative control had two isolators each. Birds were evaluated daily for 13 days and birds that were either unwilling or unable to eat and drink were euthanized with 100 mg/kg of Na pentobarbital (390 mg/ml). By day 13, all infected birds were dead or had been euthanized. A survival analysis was conducted using JMP statistical software (JMP Group Inc., San Francisco, CA, USA) to test for the effect of line on survival time at each dose. The chi-square log-rank p-values at each dose were used to compare the lines. To test for the main effects of line and dose both a parametric survival test and proportional hazard test were also conducted using JMP statistical software (JMP Group Inc., San Francisco, CA, USA).

Lentogenic challenge and RNA-seq analysis

The experimental design for the lentogenic NDV challenge has been previously described in detail (6). Briefly, three-week-old chicks were either inoculated with 10^7 EID_{50} La Sota NDV (challenged) or given PBS (nonchallenged) via an ocular-nasal route. The relatively resistant Fayoumi and relatively susceptible Leghorn were intermixed within the challenged and nonchallenged groups. Blocking for challenge status and line, at each time of

2, 6, and 10 dpi, one-third of the birds were euthanized for tissue collection. This design resulted in a total of 12 treatment groups: two challenge groups (challenged and nonchallenged), two lines (Fayoumi and Leghorn), and three time points (2, 6, and 10 dpi).

Using the RNAqueous kit Total RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) RNA was isolated from the tracheal epithelial cells, lungs, and Harderian glands of about 4 birds per treatment group. The RNA was treated with DNAse and the quality of each sample was confirmed (RQN>8). The Illumina TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA, USA) was used to generate cDNA libraries. Libraries were sequenced on the HiSeq2500 for 100 bp, single-end reads at the Iowa State University DNA Facility (Ames, IA, USA). The sequencing data was analyzed with a standard pipeline and the *Gallus-gallus* 5 reference genome (Gal5; GCA_000002315.3): FASTX, TopHat2 (25), and HTSeq (26). Transcripts with less than 4 counts across all samples were removed prior to normalization. The RNA-seq data is publicly available from ArrayExpress at EMBL-EBI under accession numbers: E-MTAB-5431, E-MTAB-5859, and E-MTAB-6038.

For data visualization, pcaExplorer (15) was used to generate PCA plots based on count data normalized using DESeq2 (27), accounting for tissue and treatment group. The variance associated with each principal component was calculated with the 1000 most variant transcripts (15).

Differential expression analysis was performed to determine how the tissues responded differently to the NDV challenge using the GLM in edgeR (28), accounting for every combination of tissue, line, dpi, and challenge status resulting in 36 levels. Within each line and dpi, the following contrasts were written to identify transcripts that were significantly impacted by the interaction between challenge and tissue: (Tissue i, Line j, dpi

k, Challenged – Tissue i, Line j, dpi k, Nonchallenged) - (Tissue i', Line j, dpi k, Challenged – Tissue i', Line j, dpi k, Nonchallenged). With three tissues, two lines, and three time points, a total of 18 contrasts were analyzed.

Co-expression analysis was performed using WGCNA (29). WGCNA clusters genes into modules based on expression levels and correlates a module's eigengene to traits of interest. All transcripts with more than 4 counts across all samples were included. Transcript counts were normalized using the variance stabilizing transformation by DESeq2 (27). The soft power threshold was set to 20 and a minimum module size of 30 was used (29). The module's eigengenes were correlated to factors of interest. Coding for the factors was as follows: Line [Fayoumi (1), Leghorn (0)], days post infection [dpi; 2 dpi (0), 6 dpi (1), 10 dpi (2)], NDV [challenged (1), nonchallenged (0)], sex [male (1), female (0)], Trachea [Trachea (1), Lung (0), Harderian gland (0)], Lung [Trachea (0), Lung (1), Harderian gland (0)], and Harderian gland [Trachea (0), Lung (0), Harderian gland (1)]. For each factor, the driver genes for a module were based on the transcripts with the highest absolute gene significance and module membership calculated by WGCNA (29). Gene significance is estimated by correlating a transcript's expression profile with a sample trait. A correlation of a module eigengene and the expression profile of each transcript is used to determine a transcript's module membership score.

GO term and network analyses were performed to ascertain the predicted function of specific modules. All transcript IDs were converted to their associated gene name using Ensembl BioMart, and NCBI and Uniprot were used to find gene names for those unidentified by Ensembl. Panther (30) was utilized for GO term overrepresentation analysis using a Bonferonni correction. Only the top-level GO terms, based on the hierarchical

labeling by Panther, were presented (Figure 5-4). Associated gene names were used as input into STRING (31) for network analysis based on protein-protein interactions described in the literature. Disconnected nodes were removed and a high confidence (0.700) was required for connection of the nodes. The nodes were colored based on MCL clustering (inflation = 3) (31).

Author Contributions

MSD: Conceptualization of RNA-seq experiment, investigation, collected samples, isolated RNA, constructed cDNA libraries, formal analysis of RNA-seq and average survival time data, methodology, data visualization, writing- original draft preparation
PJM: conceptualization of experiment and experimental design for virulent challenge animal experiment; performing challenge, and collecting data and samples for virulent challenge animal experiment; writing – reviewing and editing manuscript.
KMD: investigation – conducted virulent challenge experiment and collected samples, writing – review and editing
TO: investigation - conducted virulent challenge experiment and collected samples, writing – review and editing
DC: investigation - conducted virulent challenge experiment and collected samples, writing – review and editing
RAG: Conceptualization of experiment, Experimental design, investigation - prepared viral isolate for inoculation, writing – review and editing
JCMD: Conceptualization of experiment, Experimental design, writing – review and editing
HZ: Conceptualization of experiment, Experimental design, funding acquisition, project administration, writing – review and editing
SJL: Conceptualization of experiment, Experimental design, provided resources (genetic lines), investigation, funding acquisition, project administration, supervised analysis of data, writing – review and editing

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CHAPTER 6. DISCUSSION

This dissertation reports the first study to examine responses of Fayoumi and Leghorn inbred lines to lentogenic and velogenic Newcastle disease virus (NDV) and the first to use RNA-seq to analyze (1) the Harderian gland, (2) the lung's response to lentogenic NDV, and (3) the tracheal epithelial cells' response to lentogenic NDV *in vivo*. The objective of this dissertation research was to identify candidate genes and pathways that may be associated with resistance to NDV, and that objective has been met. The major findings from the individual tissue analyses and the combined tissue analysis have implications on the poultry industry and require future studies to answer newly arisen questions of interest. This discussion will first summarize the phenotypes and major findings from the chapters of this dissertation and then synthesize the results to highlight major implications and areas for future research.

Resistance Phenotypes

The Fayoumis and Leghorns were chosen to model resistance and susceptibility to NDV based on their previous response to other pathogens (Wang et al. 2014; Pinard-van der Laan et al. 2009; Lakshmanan, Kaiser, and Lamont 1996), however, it was unknown if this model would prove valid. This dissertation research provided multiple lines of evidence for relative resistance to NDV of the Fayoumis compared to the relatively susceptibility of the Leghorns. In this dissertation, resistance was defined as the ability of the host to interfere with the pathogen life cycle (Bishop and Stear 2003). Resistance can be quantified by measuring specific phenotypes such as viral titer, antibody titer, morbidity/mortality, shedding, etc.

These studies verified the Fayoumis' relatively resistant phenotype to both lentogenic and velogenic NDV when compared to the relatively susceptible Leghorns. Most phenotypes were measured during the lentogenic challenge, the main focus of this dissertation. The Fayoumis had significantly lower viral load in the lachrymal fluid at 6 dpi as measured by qPCR and numerically higher antibody levels at 10 dpi as measured by ELISA. Lower viral levels at 6 dpi suggest increased clearance and/or inhibition of viral replication in the Fayoumi. Increased antibody levels prevent virus from entering the cell or help identify infected cells for destruction and protect against future exposure to the pathogen. In the tracheal epithelial cells significantly less viral transcript counts were detected in the challenged Fayoumis than Leghorns at 2 dpi as measured by RNA-seq. In the Harderian gland significantly higher viral transcript counts were detected in the Fayoumis at 2 dpi, but were absent by 6 dpi, whereas the viral transcript counts were still detectable in the Leghorns at 6 dpi. We predict that the increased viral transcript counts observed in the Fayoumis at 2 dpi in the Harderian gland may be beneficial for antibody production. This is a novel method thus the correlation between viral transcript counts and viral load has not been confirmed, and the limit of detection of this method is unknown.

Once the Fayoumis' resistance to lentogenic NDV was confirmed, the major question that needed to be answered was: is the Fayoumi also relatively resistant to velogenic NDV? Trials at Southeast Poultry Research Laboratory (SEPRL) showed the Fayoumis survived longer than the Leghorns after challenge with velogenic NDV. The survival time was significantly impacted by both line and dose. Increased survival time suggests the virus took longer to overcome the host immune system. These are the only data available at this point comparing the Fayoumi and Leghorns' response to velogenic NDV.

A parallel study conducted at UC Davis provided further evidence for the Fayoumi's increased resistance when the chicks were subjected to heat stress and then challenged with lentogenic NDV. The challenged Fayoumis had significantly lower viral load at both 2 and 6 dpi than the Leghorns in the lachrymal fluid (Huaijun Zhou, personal communication). Also the Fayoumis had significantly higher antibody levels at 10 dpi than the Leghorns (Huaijun Zhou, personal communication). This suggests heat stress exacerbated the difference in relative resistance to lentogenic NDV between the lines. The current study, in combination with studies done at UC Davis and SEPRL, has established the validity of using these inbred lines as a discovery platform to characterize mechanisms of resistance to NDV.

Tracheal Epithelial Cells Summary

The RNA-seq analysis was performed on the tracheal epithelial cells only, but has been referred to as the "trachea" for simplicity. The PCA plot (data not shown) and the number of DEG showed NDV challenge had a large impact on the trachea transcriptome. As a result, the response of this tissue's transcriptome to NDV was the most intuitive and easiest to understand. The NDV challenge caused the most DEG early on and, as the chickens recovered, the challenged group became less different from the nonchallenged birds, resulting in fewer DEG as time progressed.

In both lines the DEG due to challenge at each time point predicted enrichment of immune related cells, likely a result of immune cell infiltration. The immune cell infiltration may be more pronounced in the trachea than in the lung and Harderian gland, likely due to the lack of resident lymphoid structures in the trachea (de Geus, Rebel, and Vervelde 2012). The lack of resident lymphoid structure is a possible cause for the high viral transcript counts at 2 dpi. Though the epithelial cells of the trachea have immune capabilities (Weitnauer,

Mijosek, and Dalpke 2016), the trachea relies on the migration of immune cells to clear the virus. The lentogenic virus's ability to successfully replicate in epithelial cells is also likely a major contributing factor to the high viral transcript counts in the trachea detected by RNA-seq.

Analysis of the trachea transcriptome in these inbred lines resulted in two proposed explanations for the Fayoumis relative resistance. First, compared to the nonchallenged Fayoumis, the challenged Fayoumis significantly downregulated several collagen related genes at 2 dpi in the trachea. Only 8 of the 30 collagen related genes were also DE in the Leghorns at that time. Secondly, the EIF2 signaling pathway was more activated in the challenged Fayoumis than Leghorns at 2 dpi. EIF2 signaling is an important pathway for preventing NDV replication (Zhang et al. 2014).

Overall, the challenge impacted the trachea transcriptome at all time points tested. It is impossible to know with the data at hand if expression changes were due to up or downregulation of genes, the influx of immune cells, the extensive damage that NDV causes the trachea (Kotani et al. 1987), or a combination of these factors. During tissue processing, differences in tracheal lesions between challenged and nonchallenged birds were observed. It would be valuable to specifically compare the histopathology between the two lines after challenge. This would also help to interpret the gene expression changes.

Lung Summary

The lack of detectable viral transcripts in the lung suggested that the virus was not replicating or replicating below our limit of detection at the time points measured. The presumed lack of virus in the lung may be a major reason the PCA was unable to separate individuals based on challenge and may have caused the unexpected pattern of DEG numbers

between the challenged and nonchallenged birds over time. At all time points the Leghorns had few DEG in response to the challenge suggesting non-responsiveness, however, their were larger differences between the challenged and nonchallenged Fayoumi lung transcriptomes.

The number of DEG at earlier time points were few (<102), but the Fayoumis at 10 dpi had over 2,500 DEG between challenged and nonchallenged birds. Many of these DEG resulted in the activation and inhibition of several immune pathways. In response to infection the EIF2 signaling pathway should be activated to inhibit viral replication (Zhang et al. 2014). However, this pathway was inhibited in the challenged Fayoumis at 10 dpi. At first this result was unexpected, but in the absence of virus, inhibiting viral replication would not be necessary. Thus in order to understand the tissue's response to infection it is crucial to know whether the pathogen is concurrently replicating in said tissue.

The lung transcriptome of the nonchallenged Fayoumis changed over time, more than the Leghorns or challenged birds of either line as measured by DEG. We have predicted that the changes observed in the nonchallenged birds over time were not observed in the challenged Fayoumi lungs over time due to resource allocation (Rauw 2012). Thus the challenge may have been responsible for disrupting normal lung development. Resources were likely allocated to fighting off the infection in other tissues and/or the immune cells that would have established the resident lymphoid structures of the lung under nonchallenged conditions were "re-purposed" towards fighting off infection. The DEG over time in the nonchallenged Fayoumis may be related to faster/earlier development of bronchus associated lymphoid tissue (BALT) or resident lymphoid tissue compared to the Leghorns (de Geus, Rebel, and Vervelde 2012). More rapid development of the resident lymphoid structures

would improve the Fayoumis' ability to combat respiratory pathogens at an earlier age. It could also result in a better response to aerosol delivered vaccines. The analysis of the lung thus provided new information about lung development from 23-31 days of age.

The changes in the nonchallenged Fayoumis over time made the interpretation of the differential expression analysis more difficult. Co-expression analysis did not rely on the contrasts between age-matched, but perhaps not developmentally equivalent, challenged and nonchallenged birds. Co-expression analysis proposed candidate modules and driver genes. The genes within the black module, including cNFI-1, RHOT2, UBE2E3, and CDC5L, were associated with line, viral load, and antibody levels. These results may provide good targets for selection for disease resistance. Increased phenotypes, including pathology scores, lung weight, histology etc. would have been beneficial for this analysis.

Harderian Gland Summary

This was the first time the Harderian gland had ever been analyzed with RNA-seq. For this reason the data were further analyzed to characterize the Harderian gland's basal expression and compare it to other immune tissues in order to determine what makes the Harderian gland unique compared to other chicken immune tissues (Appendix 1).

Overall, the challenge did not have a large impact on the Harderian gland transcriptome, as determined by DEG, with one exception. The Leghorns at 6 dpi had many DEG. Pathway analysis suggested the Leghorns at this time were activating both the cell mediated and humoral arms of the adaptive immune response. The large number of DEG in the Leghorn at 6 dpi may correspond to the viral transcript counts still detectible at this time, especially since DEG at this time were predicted to inhibit viral replication.

Contrasting the Fayoumi and Leghorns within each challenge group and time showed on average approximately 320 DEG for each contrast not including the challenged Fayoumi vs. challenged Leghorn contrast at 2 dpi, which resulted in 2,298 DEG. The high number of DEG indicated the Fayoumi and Leghorn Harderian glands were the most different in the challenged birds at 2 dpi. Therefore, the two lines likely differed in their early response to the virus, but this response was not enough to detect DEG in response to challenge within lines. Pathway analysis of the DEG between the two lines showed the nonchallenged Fayoumis might have a more primed immune system compared to the Leghorns, because several immune related pathways were more activated in the nonchallenged Fayoumis than Leghorns. The primed immune system may have eliminated the virus without needing to further activate the immune system, which may be why the Fayoumis had few DEG in response to challenge. Histology and RNA-seq from an earlier time point post challenge would provide valuable information about how this unique tissue immediately responds to NDV.

Combined Tissue Summary

Analysis using all three tissues provided the most comprehensive picture of the whole animal response to NDV infection. Differential expression analysis requires contrasts to compare groups. With 3 tissues, 2 lines, 2 challenge groups, and 3 time points, the extreme number of contrasts required to analyze the complex data meant this method was inadequate for a comprehensive analysis. The extreme number of contrasts, unique pattern in numbers of DEG within each tissue, and differences in viral load among tissues led to the use WGCNA to jointly analyze all three tissues.

The trachea, lung, and Harderian gland are distinct tissues with different functions under homeostatic conditions and they had distinct responses to viral challenge. Analyzing the tissues concurrently provided new insights. The tissues clearly separated in the PCA plot, and WGCNA showed that many clusters of genes (modules) were significantly correlated with the tissues, both indicating tissue specific patterns in gene expression. Three modules were of particular interest due to their relationship with the challenge and/or line. GO term and network analyses showed the genes within these modules have important immune functions. EIF2AK2, MPEG1, and TNFSF13B were driver genes for modules of interest and therefore were proposed as candidate genes important for the host response to NDV. To the author's knowledge using WGCNA was a novel and unique way to analyze the host response to stimulus in multiple tissues. Analyzing the trachea, lung, and Harderian gland together provided a more inclusive representation of the whole animal's response than analyzing the tissues individually.

Overall Synthesis

The Fayoumis are relatively resistant to Newcastle disease virus compared to the Leghorns.

The phenotypes collected for both the lentogenic and velogenic challenges showed that the Fayoumis had a more favorable response to the virus than the Leghorns. For the Harderian gland viral load, however, this result was not as clear. Because the viral transcript counts were higher in the Fayoumis than Leghorns at 2 dpi in the Harderian gland, this introduces the opportunity for interesting speculations about (1) the interpretation of viral load as a resistance phenotype and (2) whether relative resistance and susceptibility is tissue dependent. We hypothesize the increased viral transcript counts at 2 dpi in the Fayoumis were beneficial for inducing neutralizing antibody production, because the F and HN viral

transcripts were detected at high levels, and transcripts for the P and L proteins, which are required for NDV replication, were at low levels. Antibodies directed at these outer viral proteins could prevent entry into the host cell and mark the virus for destruction. Measuring neutralizing IgA in the tears and/or IgY and IgM antibodies in the Harderian gland could provide evidence that the increased viral transcript counts at 2 dpi in the Fayoumi may to lead to more neutralizing antibodies. However, this mechanism would need to be tightly controlled because increased NDV load within a tissue was previously associated with increased pathology and inflammation (Hu, Hu, et al. 2015), which would not be ideal for the chicken. Establishing the Fayoumis and Leghorns as a discovery platform for modeling NDV resistance was a very important outcome of this dissertation study.

Viral transcript counts likely had a large impact on the tissues response to challenge.

It was very clear that each tissue responded uniquely to the NDV challenge in terms of DEG, and this may be due in part to the differences in viral transcript counts within each tissue. T cell related pathways were activated only in tissues with detectable virus. Several T cell pathways activated in the Harderian gland and the trachea, but were not activated in the lung. The cell-mediated immune response is very important in reducing shedding and clearing NDV (Russell, Dwivedi, and Davison 1997; Kapczynski, Afonso, and Miller 2013; Marino and Hanson 1987).

Although there were detectable viral transcripts in the Harderian gland, viral transcript counts were much higher in the trachea. PCA plots showed challenge had the biggest impact on the trachea transcriptome, whereas line had the biggest impact on the lung and Harderian gland. The higher the viral transcript counts detected in each tissue, the larger the impact on the transcriptome (quantified by PCA or differential expression). Viral

transcripts were likely the highest in the trachea due to tissue composition. La Sota NDV replicates well in epithelial cells (Alexander and Senne 2008), and because the RNA was isolated from the tracheal epithelial cells only, this may have resulted in a high concentration of virus. Viral transcript counts may have been lower in the Harderian gland than the trachea due to the large number of immune cells stationed in the Harderian gland under resting conditions (Bang, Foard, and Bang 1974).

Gene expression is impacted by many factors

Whole-genome bisulfite sequencing and RNA-sequencing data from the Fayoumi and Leghorn lungs suggests DNA methylation played a role in gene expression and transcription stability (Li et al. 2015). It is known that tissue (Sun et al. 2016), genetic line (Wang et al. 2014), viral strain (Liu et al. 2012; Hu, Hu, et al. 2015), copy number variants (Abernathy et al. 2014), and many other factors can impact gene expression levels. Tissue samples are heterogeneous mixtures of multiple cell types; therefore, we are unable to differentiate between differential expression and differential cell composition. It is important to focus on pathways and themes that were more conserved across tissues and strains. In this discussion, pathways and genes that are impacted across multiple tissues by genetic line or challenge were considered.

Genes most often differentially expressed

Within each tissue 15 contrasts were analyzed comparing the lines within challenge group and time, the challenge groups within line and time, and the interaction between line and challenge group within time. With three tissues, this resulted in 45 total contrasts. To identify potential candidate genes the genes were ranked by the number of times they were differentially expressed. The top ranked transcript was ENSGALT00000047912, a novel protein coding gene that was DE in 21 out of the 45 contrasts. This transcript was always

more highly expressed in the Leghorns within each challenge group and at each dpi in all three tissues. Furthermore, this transcript was upregulated in response to challenge in both lines at 2 dpi in the trachea and downregulated due to challenge in the Fayoumis at 10 dpi in the lung.

Two transcripts were DE in 20 of the 45 contrasts, Tryptophan 2,3-dioxygenase (TDO2) & MHC class I alpha chain 2 (BF2). TDO2 knockdown results in T cell proliferation in human tumors (Opitz et al. 2011). Therefore lower expression of TDO2 may increase the number T cells, which are key in host defense against NDV. In the current study, TDO2 was always more highly expressed in Leghorns than Fayoumis in all contrasts and downregulated after challenge in the Leghorns at 2 dpi in the trachea and in the Leghorns at 6 dpi in the Harderian gland. The lower expression of TDO2 in the Fayoumis may correspond to increased T cell proliferation relative to the Leghorns. BF2 is responsible for displaying intracellularly derived peptides on the cell surface for detection by CD8+ T cells; if BF2 displays viral proteins this will result in death of the infected cell. This protein is key in response to all intracellular pathogens and is crucial in bridging the innate and adaptive immune system. BF2 was more highly expressed in the Fayoumis compared to the Leghorns at all times and in all challenge groups in the trachea and lung, and in the challenged and nonchallenged birds at 2 dpi and the nonchallenged birds at 6 dpi in the Harderian gland. BF2 was also upregulated in response to challenge in both lines at 2 and 6 dpi in the trachea, and in the Leghorns at 6 dpi in the Harderian gland. The expression of both TDO2 and BF2 may be related to the Fayoumi's resistant phenotype.

EIF2 signaling

One pathway of particular interest was the EIF2 signaling pathway because of its known impact on NDV replication (Zhang et al. 2014). Under normal conditions this pathway is important for protein translation. Within this pathway EIF2AK2 (also known as PKR) is activated by dsRNA, which is produced during NDV transcription (Zhang et al. 2014). The activated EIF2AK2 phosphorylates eIF2α (Zhang et al. 2014). eIF2α is necessary for NDV replication, but once it is phosphorylated by EIF2AK2, eIF2α has antiviral effects (Zhang et al. 2014). When the Fayoumis and Leghorns were directly compared, this pathway was more activated in the challenged Fayoumis at 2 dpi in the trachea and more activated in the nonchallenged Fayoumis at 10 dpi in the lung. At 10 dpi in the Fayoumis, the EIF2 signaling pathway was inhibited in the challenged compared to the nonchallenged birds in the lung. In the spleen, EIF2 signaling pathway was relatively activated in the challenged Fayoumis compared to the challenged Leghorns at 2 dpi (Zhang et al. 2018). A related pathway, Role of PKR in interferon induction and antiviral response, was significantly impacted by challenge in the Leghorns at 6 dpi in the Harderian gland and in both lines at 2 dpi in Trachea; however, the z-score in all instances was 0, meaning IPA could not predict activation or inhibition based on the expression of genes within the pathway. Additionally, role of PKR in interferon induction and antiviral response was significantly impacted due to challenge in both lines in the spleen at 2 dpi (Zhang et al. 2018). These pathways may be associated with NDV resistance.

Furthermore, EIF2AK2 was a driver gene from the darkolivegreen module for challenge status in the combined tissue analysis. EIF2AK2 was significantly upregulated due to challenge in the Leghorns and Fayoumis at 2 dpi in both the trachea and spleen (Zhang et

al. 2018). Previous studies have shown knockdown of EIF2AK2 resulted in increased NDV titer and overexpression of EIF2AK2 resulted in decreased viral titer (Zhang et al. 2014). This pathway has a role in host defense to both lentogenic and velogenic strains of NDV. Both La Sota and a velogenic NDV strain activated the EIF2 signaling pathway in HeLa cells (Zhang et al. 2014). Expression analysis of the spleen showed significant upregulation of EIF2AK2 after challenge with virulent NDV at 1 and 2 dpi (Rue et al. 2011). The EIF2AK2 protein is involved in various immune pathways and processes including three TLR pathways, STAT, NF-κβ, IRF-1, and more (Garcia et al. 2006). Chickens lack the RIG-I protein that is responsible for detecting RNA viruses (Barber et al. 2010). In the absence of RIG-I, the utilization of EIF2AK2 as a pattern recognition receptor to detect dsRNA from viruses may be even more crucial in chickens.

Collagen

The downregulation of nearly 30 collagen related genes in the trachea and downregulation of COL5A1 in the lung due to challenge in the Fayoumis at 2 dpi was an unexpected result that may be related to the Fayoumis resistant phenotype. Collagen related genes were DE in many contrasts especially in the trachea and lung. For example, COL19A1 was upregulated in the nonchallenged Fayoumis compared to the Leghorns at 2 dpi in the lung, COL26A1 was significantly downregulated at 6 dpi in the Leghorns due to challenge in the trachea, and COL4A2 was downregulated in the nonchallenged Fayoumis compared to the Leghorns at 6 and 10 dpi in the lung. Collagen is a major component of the extracellular matrix, and changes in the extracellular matrix have major consequences. The migration of immune cells is greatly influenced by the cellular environment (Lammermann and Germain 2014). There are important interactions between collagen and T cells for their activation,

proliferation, survival, and retention in peripheral tissues (DeNucci, Mitchell, and Shimizu 2009; Ray et al. 2004; Fiorucci et al. 2002). Also, apoptosis may increase with collagen downregulation due to anoikis (method of apoptosis that occurs when the epithelial cells and collagen detach) (Frisch and Francis 1994). Apoptosis of viral infected cells is a host mechanism for preventing spread of the pathogen. The downregulation of collagen may be beneficial for defense against other chicken viruses as well (Smith et al. 2015). This may be the first study to indicate the important role of collagen in the chicken's response to NDV infection.

mTOR signaling

mTOR signaling is an important pathway involved in host stress response and results in autophagy (Watanabe, Wei, and Huang 2011). Autophagy is disadvantageous for some replicating viruses, because viral proteins are more likely to be sequestered and destroyed (Le Sage et al. 2016), but for other viruses (like NDV) autophagy increases viral replication (Sun et al. 2014). Previous studies suggest mTOR signaling may have a role in tolerance to NDV (Susta et al. 2016). The mTOR pathway was significantly impacted in the challenged Fayoumi vs. Leghorn contrast at 2 dpi in the spleen (Zhang et al. 2018). Interestingly, the mTOR pathway was activated due to challenge in the Fayoumis at 10 dpi in the lung, and RICTOR a major component of the mTOR complex was upregulated in the Fayoumis at 10 dpi due to challenge. Also, the expression levels of genes within the mTOR pathway in the challenged Fayoumis at 10 dpi were predicted to result in autophagy. Although the mTOR pathway seemed to be most important in tissues (spleen & lung) that may have had little to no direct exposure to the virus, RICTOR was identified as a top driver gene for line in the steelblue module in the combined tissue analysis. This suggests the two lines may differ in in

this important pathway, although differences in the mTOR pathway between the lines were not detected elsewhere. NDV is known to promote autophagy via the mTOR pathway in lung cancer cells (Hu, Sun, et al. 2015), and therefore, it was surprising this pathway was not significantly impacted in the tissues with detectable viral transcripts.

TNFSF13B / TNFRSF13B

In chickens, TNFSF13B (also known as BAFF) is important for the survival and proliferation of B cells (Schneider 2004). In the combined tissue analysis TNFSF13B was a driver gene for challenge status from the darkred module, suggesting the expression of this gene was highly correlated with challenge and may have influenced the expression levels of other genes within the darkred module. In the trachea, TNFRSF13B (a receptor for TNFSF13B) was significantly impacted by the challenge by line interaction at 6 dpi in the trachea. A separate study showed TNFRSF13B was differentially expressed in the lung of Fayoumis and Leghorns challenged with AIV (Wang et al. 2014), and TNFSF13B was upregulated in the spleen after challenge with velogenic NDV (Rasoli et al. 2014). Also, a genome wide association study proposed TNFRSF13B as a candidate gene for the chicken's response to lipoteichoic acid (LTA) a cell wall component of gram-positive bacteria (Siwek et al. 2015). Both TNFSF13B and TNFRSF13B may be associated with disease resistance to multiple pathogens and deserve further attention.

Implications / Future Directions

The objective of this study was to identify candidate genes and pathways associated with resistance to NDV, and that objective has been met. Collagen, EIF2 signaling, mTOR signaling, TNFSF13B/TNFRSF13B, TDO2, and BF2 represent mechanisms, pathways, and genes involved in the early and late response to NDV that have the potential to regulate host resistance to NDV. These results have the ability to improve vaccination strategies, influence

breeding strategies, and better understand the host response to viral pathogens as described below.

Vaccination

The La Sota vaccine has shown to confer 100% protection against mortality after challenge with virulent strains of NDV, if there is enough time between vaccination and challenge and if the vaccination is given at the proper age, however, the vaccine does not prevent virus shedding (Dimitrov et al. 2017). Vaccination strategies can be improved by the findings of this dissertation study. It is well known that eye-drop/aerosol vaccines provide better results than intramuscular vaccines (Ewert, Barger, and Eidson 1979) because they stimulate the local mucosal immunity. This study provided novel evidence on what genes and pathways were important in stimulating the local immune response in the Harderian gland as well as the lung and trachea. Reverse genetics can be used to modify NDV (Dortmans et al. 2011). Recombinant vaccines incorporating genes of interest discovered in this study could be used to increase antibody levels or reduce shedding. Previous studies have shown co-administration of the NDV vaccine and an IL18 recombinant plasmid improved the response to vaccine (Wang et al. 2015), however, using IFN-γ as an adjuvant did not improve the response to vaccine (Cardenas-Garcia et al. 2016). Multiple genes identified in this dissertation study could be tested for use as DNA or protein adjuvants. This research clearly showed the response to vaccine (viral load, antibody levels, DEG) was dependent on host genetics. Alteration of the vaccine schedule, dosage, or the vaccine itself based on host genetics may provide better results. Or, it may be possible to select chickens based on their response to vaccine, in order to generate more “vaccine-ready” birds.

Breeding Strategies

This study is part of a USAID funded project that aims to breed chickens for increased resistance to NDV. Many genomic wide association studies (GWAS) have been performed on multiple breeds and their response to both lentogenic and velogenic NDV. Early evidence suggests resistance phenotypes like viral load and antibody levels in response to lentogenic NDV are moderately heritable and controlled by many genes (Rowland et al. 2016; Rowland et al. 2017). Few SNPs have been shown to be significant in the GWAS done on the Hy-line brown lines (Rowland et al. 2017). Software like Camoco can be used to integrate expression and GWAS data (Schaefer et al. 2017). Also, the differentially expressed genes identified in this study can add confidence to local SNPs that may be weakly associated with a resistance phenotype.

Another beneficial aspect of the differential expression analysis is to provide evidence for candidate genes located near a significant SNP. If a significant SNP associated with viral load was found near several genes and one of those genes was DE after challenge, this suggests that the DE gene is a stronger candidate. Knowing the causal mutation or which gene is impacted can allow breeders to better predict the effect of selection on said SNP. Selection on SNPs associated with resistance without knowing the pleiotropic effects of that QTL could have unforeseen negative impacts. Multiple studies will need to be performed, because mechanisms of resistance should be understood prior to selection. Another reason for the necessity of understanding mechanisms of resistance prior to selection is to prevent the improvement of resistance to one disease at the detriment to another (Pinard-van der Laan, Siegel, and Lamont 1998), or to the detriment of other economically important traits.

Low heritability of NDV traits and few significant SNPs that account for a small portion of the variance associated with the traits of interest impact the sustainability and efficiency of SNP based selection. Gene expression is highly heritable (Dermitzakis 2008) and may be a better strategy for increasing host resistance. Selection based on gene expression has had success (Swaggerty et al. 2008). However, early GWAS results have shown that NDV resistance (as measured by viral load and antibody levels) is an infinitesimal trait, and no major QTLs have been discovered (Rowland et al. 2017). This lack of significant SNPs suggests that it may be unlikely that the expression of one gene could be used as a biomarker, unless that RNA was a driver gene, a gene whose expression impacts a network of genes identified in the WGCNA analysis. We know that the two lines differ in the expression of immune related genes under nonchallenged conditions at an early age. If the expression levels of a set of genes at an early age can predict a beneficial outcome, this would be ideal. The blue module from the lung study and the lightyellow module from the combined tissue analysis may include some excellent candidate genes. Both these modules were highly correlated with line and the blue module genes were associated with several immune related GO terms. A separate study has compared the expression levels of innate immune genes in response to NDV *in ovo* in the Fayoumis and Leghorns and other breeds to identify biomarkers associated with resistance (Schilling et al. 2018). If information on half-sibs could be collected prior to hatch it would save money and increase the rate of genetic progress. If wanting to select on breeder phenotypes, more studies need to be done on the blood transcriptome or using tracheal swabs to identify potential RNA or protein biomarkers that can be easily measured directly on an individual without euthanizing. eQTL studies

across multiple ecotypes to look for non-breed specific SNPs that impact expression of important driver genes that are also associated with beneficial phenotypes would be ideal.

Future studies

This study included three important phenotypes to quantify relative resistance, viral and antibody load and mean survival time. Future studies need to include more phenotypes to better understand mechanisms of resistance including: viral shedding, neutralizing antibody levels (Chumbe et al. 2017), lesion scores, antibody levels over time, and production traits to uncover the genetic relationships between the immune system and production traits. Immune response traits may vary dependent on infection strain, genetic lines, host age, etc. The bone marrow derived dendritic cells of the Fayoumis and Leghorns have been previously analyzed for abundance of specific cell surface proteins and phagocytic ability (Van Goor et al. 2016). It may be useful to characterize the Fayoumi and Leghorn bone marrow derived dendritic cells in response to multiple NDV infection strains. Additionally, methods like thiouracil cross-linking mass spectrometry (TUX-MS) could be used to identify host proteins that directly interact with the virus (Lenarcic et al. 2013). It would be interesting to see if the Fayoumis and Leghorns differed in the abundance or type of proteins that directly bind to NDV.

One concern for selecting birds resistant to lentogenic NDV is that in a backyard setting when chickens are exposed to lentogenic NDV, a chicken with increased resistance may not become infected and will lose this opportunity for “natural” vaccination. Then this resistant chicken with no antibodies to defend against velogenic NDV will likely die if exposed. While a more susceptible bird that was infected with lentogenic NDV and produced

antibodies, may survive the exposure to velogenic NDV. This may be one reason that natural selection is not enough to increase resistance.

Although RNA-seq is a useful tool, the method used in this dissertation only measured the polyadenylated RNA levels; there are many other types of RNA (miRNA, siRNA, piRNA, eRNA, etc.) that may be important to NDV resistance. We also assume a correlation between mRNA levels and protein expression, but this might not always be the case. Protein abundance, cellular localization, and post-translational modifications should also be studied. Most information on the functional genomics analyses used in this study comes from human/mouse/rat studies. We know there are distinct differences in the immune system of chickens; therefore, confirmation of our hypotheses in chickens is necessary. Using CRISPR for gene knockouts or insertions would be an ideal tool for providing definitive evidence for the function of our candidate genes of interest in regards to NDV. Previous studies have selected stem cells for NDV tolerance (Susta et al. 2016). Those cells can be used as primordial germ cells and transferred into a sterile chicken to produce offspring. Experimental lines created by genetic engineering would be beneficial for increasing fundamental knowledge. It is possible in the future that genetic engineering to prevent disease may be allowed in commercial production. The industry should be prepared for that day.

Conclusion

Comprehensive understanding of the host response to NDV has been improved by this study. Additionally, the Harderian gland is better characterized (see Appendix I). Overall, EIF2 signaling, collagen, mTOR signaling, TDO2, BF2, and TNFSF13B/ TNFRSF13B are pathways and genes that were identified as significant in multiple

comparisons in this study may play crucial roles in resistance towards NDV. Some pathways were not obvious targets from the start of the experiment, which is why using RNA-seq was so useful. This research enhances the understanding of the host response to NDV and provides candidate genes and pathways that may be related to resistance. Gaining a better fundamental understanding of how the chicken responds to disease is vital if improvements in resistance are to be made efficiently. This study also identified several unknown or novel proteins and lincRNAs associated with the host response to NDV. Overall, these findings direct further research to uncover the function of these novel elements and known pathways in regards to NDV resistance.

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APPENDIX [WHAT MAKES THE HARDERIAN GLAND TRANSCRIPTOME DIFFERENT FROM OTHER CHICKEN IMMUNE TISSUES? A GENE EXPRESSION COMPARATIVE ANALYSIS]

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Abstract

The Harderian gland is a sparsely characterized immune tissue known to play an important role in local immunity. The function of the Harderian gland, however, is not clearly defined. Measuring the expression of all genes using RNA-seq enables the identification of genes, pathways, or networks of interest. Our relative RNA-seq expression analysis compared the chicken Harderian gland transcriptome to other important primary and secondary immune tissues including the bursa of Fabricius, thymus, and spleen of nonchallenged birds. A total of 2,386 transcripts were identified as highly expressed in the Harderian gland. Gene set enrichment showed the importance of G-protein coupled receptor signaling and several immune pathways. Among the genes highly expressed in the Harderian gland were 48 miRNAs, a category of genetic elements involved in regulation of gene expression. Several identified miRNAs have immune related functions. This analysis gives insight to the unique immune processes inherent in the Harderian gland.

Introduction

Avian species have many unique immunological features compared to mammals with whom they last shared a common ancestor over 310 million years ago (Hedges, 2002). In birds the spleen is the largest lymphoid tissue, but is only able to encounter antigens that circulate through the blood because unlike mammals, birds lack a lymphatic system (Oláh et

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al., 2013). T cell development is similar in mammals and birds, but chickens have more γδ T cells than humans (Smith and Göbel, 2013). The thymus is a primary immune tissue where T cell development, differentiation, and maturation occurs.

Humans completely lack the bursa of Fabricius and only have a rudimentary Harderian gland, whereas these two tissues play very important roles in the chicken immune system. The bursa is a unique primary immune organ found in birds that plays a critical role in the immune response. B cell development, proliferation, and diversification occurs in the bursa, where B cells also undergo immunoglobulin rearrangement to create B cell receptors and mature B cells (Glick et al., 1956). The Harderian gland is located behind the eyes of the chicken and its function is not clearly defined, but includes the lubrication of the nictitating membrane (Bang and Bang, 1968). It is a relatively small tissue; in adult chickens the average weight was found to be 84.4 mg (Wight et al., 1971). The Harderian gland is known to contain many B cells. The majority of cells within the Harderian gland react to anti-chicken bursa cell serum (Albini and Wick, 1974). Lymphocytes from the bursa migrate to the Harderian gland prior to hatch and may not be involved in systemic immunity (Mueller et al., 1971; Baba et al., 1988). Also, terminal B cell maturation may occur in the Harderian gland (Manisikka et al., 1989). The Harderian gland is also home to large numbers of T cells. Equal numbers of CD3+ and Bu-1+ cells were found in the Harderian gland of both control and vaccinated chicks, and there were twice as many CD4+ than CD8+ cells in unvaccinated chicks as measured by immunostaining using monoclonal antibodies (Russell et al., 1997). The B and T cells of the Harderian gland play an important role in local immunity (Manisikka et al., 1989; Maslak, 1994). The bursa, thymus, spleen, and Harderian gland are among the most important immune tissues in the chicken.

Previously, these four immune tissues were compared directly via immunohistochemistry staining. In ducks, induction of CD8+ cell immunity within the spleen and thymus was stronger after challenge with an attenuated strain of hepatitis A, whereas in the bursa and Harderian gland CD8+ cell immunity was induced more strongly after challenge with the virulent strain (Ou et al., 2017). These tissues respond differently to antigen. In unstimulated chickens, μ Heavy chain and λ Light chain mRNA were expressed higher in the Harderian gland than the bursa, spleen, and thymus (Manisikka et al., 1989). Studying the transcriptome of these tissues elucidates the mechanisms utilized in response to pathogens. Until recently, the Harderian gland transcriptome had never been analyzed (Lamont, personal communication).

Unlike the Harderian gland, the transcriptomes of the bursa, spleen, and thymus tissues are well characterized. Transcriptome analysis of the bursa revealed BCR receptor signaling, cytokine-cytokine receptor interaction, lysosome, CAM, and apoptosis pathways were impacted by avian pathogenic *E. coli* (APEC) (Sun et al., 2015), apoptosis of IgM+ cells, infiltration of macrophages, and increased expression of pro-inflammatory genes were seen after infection with velogenic Newcastle disease virus (NDV) (Kristeen-Teo et al., 2017), and defense response to virus, positive regulation of T cell-mediated cytotoxicity, and *IFN-γ* production pathways were impacted by infectious bursal disease virus (IBDV) infection (Ou et al., 2017). A combined heat and lipopolysaccharide (LPS) stress event showed the bursa transcriptome decreased expression of Wnt signaling genes and increased leukocyte migration and activation (Lamont, personal communication).

The spleen transcriptome responded to a combined heat stress and LPS treatment by altering the expression of genes within the Hepatic Fibrosis/Hepatic Stellate Cell Activation

and Macrophages, Fibroblasts, and Endothelial Cells in Rheumatoid Arthritis pathways in two distinct genetic lines (Van Goor et al., 2017). In response to APEC infection, broiler splenic gene expression was predicted to affect the Jak-STAT and cytokine-cytokine receptor signaling pathway (Sandford et al., 2011), and the spleen responded to NDV challenge by activating interferon-stimulated genes (Zhang et al., 2018).

Compared to the bursa and spleen, there have been relatively few RNA-seq studies conducted on the chicken thymus. The thymus transcriptome responded to APEC by impacting the TLR signaling pathway, lysosome pathway, CAMs, and TCR signaling pathway (Sun et al., 2016). Another study showed thymus atrophy and its possible relationship with the expression of immune genes after challenge with LPS and *Salmonella* (Huang et al., 2016). In response to heat stress and an LPS challenge in the thymus transcriptome, ILK Signaling, Integrin Signaling, and cell proliferation pathways were all impacted (Lamont, personal communication).

Within each immune tissue, pathogen, strain, dose, time, genetic line, and more, greatly impact gene expression. Under basal conditions it is unclear how these tissues' transcriptomes compare, especially how they compare to the Harderian gland. A relative expression analysis of these fundamental immune tissues will help to better characterize the Harderian gland by identifying genes highly expressed (relative expression value greater than 2 standard deviations from the mean) in this tissue relative to the bursa, thymus, and spleen. We assume the genes highly expressed in the Harderian gland are either related to tissue-specific non-immune function of the gland, or related to the unique immune function of this tissue in contrast to the other immune tissues studied. We hypothesize that the Harderian gland has mechanisms of defense that can be triggered rapidly because of its role in local

immunity compared to the other more systemic immune tissues, and that the functional analysis of the genes highly expressed in the Harderian gland compared to the bursa, thymus, and spleen may elucidate these mechanisms.

Methods

Sample descriptions and processing

The Fayoumis (Line M 15.2) from the Iowa State University Poultry Farm (Ames, IA) have been maintained as an inbred line since 1954 resulting in an inbreeding coefficient of 99.95% (Fleming et al., 2016). All publically available RNA-seq data comes from the Fayoumi controls from either a NDV challenge experiment (Lamont, personal communication) (Zhang et al., 2018) or a combined heat stress and LPS experiment (Van Goor et al., 2017) (Table 6-1). In both experiments the Fayoumis were raised in floor pens with wood chips and *ad libitum* access to food and water. Although performed in separate batches, all tissues were collected and placed into RNAlater solution (Thermo Fisher Scientific, Waltham, MA) for short-term storage, tissues were homogenized using mechanical disruption, RNA was isolated using an RNAqueous kit (Thermo Fisher Scientific, Waltham, MA), DNAse treated with the DNA-free kit (Thermo Fisher Scientific, Waltham, MA), and assessed for quality (RQN or RIN>8). All samples underwent the same protocols to generate the cDNA libraries (TruSeq RNA sample preparation guide (v2; Illumina, San Diego, CA)), and were sequenced on the same HiSeq2500 machine to generate 100 bp single-end reads at the Iowa State University DNA Facility (Ames, IA) (Table 6-1). From the NDV experiment at ages 23 and 27 days, three of the four spleen and Harderian gland tissue samples were from the same individual birds, and the spleen, thymus, and bursa samples from the heat/ LPS experiment came from the same four individuals.

Table 6-1: Sample information

Tissue	Age (days)	Number of birds	Male:Female	Accession[c]
Harderian gland[a]	23	4	2:2	E-MTAB-6038
Harderian gland[a]	27	4	3:1	E-MTAB-6038
Harderian gland[a]	31	4	2:2	E-MTAB-6038
Spleen[a]	23	4	2:2	E-MTAB-5851
Spleen[a]	27	4	3:1	E-MTAB-5851
Spleen[b]	22	4	1:3	GSE85434 (GEO)
Thymus[b]	22	4	1:3	E-MTAB-6290
Bursa[b]	22	4	1:3	E-MTAB-6289

[a]Fayoumi controls from an Newcastle disease virus challenge experiment
[b]Fayoumi controls from a heat stress/LPS experiment
[c]Data sets available from ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) or GEO (https://www.ncbi.nlm.nih.gov/geo)

The RNA-seq data underwent a standard pipeline previously described (Deist et al., 2017) and was mapped to the Gallus_gallus-5.0 (GCA_000002315.3) reference genome using TopHat2 (Kim et al., 2013). The number of reads mapped to each transcript was counted using HTSeq (Anders et al., 2015). All transcripts with less than four counts across all samples were removed resulting in 18,123 of 38,118 usable transcripts.

Calculating relative expression values

The protocol for calculating relative expression values was previously described (Bailey et al., 2009; Pritchett et al., 2017). Pritchett et al. used the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) normalization method to normalize their counts (Pritchett et al., 2017). The current study has adapted the method using count data normalized with the variance stabilizing transformation in DESeq2 (fittype=mean; blind=true) (Love et al., 2014). A constant (2.32) was added to all normalized counts to make all values positive. The following formula was used to calculate the relative expression values (rEx).

$$rEx = log_2 \left(\frac{Maximum\ normalized\ counts\ for\ each\ transcript\ in\ the\ Harderian\ gland}{Median\ normalized\ count\ for\ each\ transcript\ in\ other\ immune\ tissues} \right)$$

A transcript's rEx value was considered significant if it was more than two standard deviations from the mean. The same significance threshold was used previously (Pritchett et al.). Comparing the maximum value to the median emphasizes the identification of transcripts highly expressed in the Harderian gland. Although this method may be sensitive to outliers, the standard deviation of individuals' normalized counts within each transcript in the Harderian gland was on average 1.05 (maximum SD=5.07, minimum=2.26E-19). The individual sample from which the maximum normalized count value was obtained was well represented across all samples. Each Harderian gland sample contributed a maximum normalized count value for at least 1,324 transcripts and at most 3,859 transcripts.

Analysis of relative expression data

For data visualization, pcaExplorer (Marini, 2016) PCA plots were generated using dds and vst normalization from DESeq2 (Love et al., 2014) accounting for tissue and individual. The top 1000 most variant transcripts were used to calculate the principal components.

Transcripts highly expressed in the Harderian gland were further analyzed using Panther (Mi et al., 2017), Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA), and STRING (Szklarczyk et al., 2017). These transcripts were converted to their associated gene name using BioMart on Ensembl (version 89) and input into Panther. Panther recognized 757 of the 992 input genes for an overrepresentation test using the GO biological process complete annotation set and the *Gallus gallus* reference list with a Bonferonni correction for multiple testing. Ensembl transcript identifiers (IDs) and the relative expression values for the transcripts highly expressed in the Harderian gland were used as

input to IPA. Of the 2,386 transcripts, 942 were mapped (identified) by IPA and used for analysis. Several canonical pathways were identified as significant, and those pathways with p-values less than 0.05 and included more than 5 genes have been reported. Associated gene names of transcripts highly expressed in the Harderian gland were input into STRING and used to generate a network. A high confidence (0.700) was used, all unconnected nodes were removed, and MCL clustering (inflation parameter = 3) was performed. A total of 836 nodes and 669 edges were included.

Results

Principal component analysis

Samples clustered very tightly by tissue (Figure 6-1). Bursa, spleen, and thymus samples from one experiment came from the same 4 birds, and there was also overlap between the birds that contributed the Harderian gland and spleen samples from the other experiment (Table 6-1). However, no clustering by individual bird was observed. Also, samples did not cluster by age or experiment. The large first principal component (70.3%) separated the Harderian gland from the other three tissues, showing the distinctiveness of the Harderian gland transcriptome. The second principal component separated the bursa, thymus, and spleen (PC2 = 13.9%). The two primary immune tissues, bursa and thymus, clustered more closely than the spleen (Figure 6-1). The spleen samples clustered very tightly together within group even though they were different ages and from different experiments (Figure 6-1, Table 6-1). Six of the ten top loading genes for principal component 1 were also identified as highly expressed in the Harderian gland and included: *MYL1*, *MYL2*, *CKMT2*, and *TMEM182*.

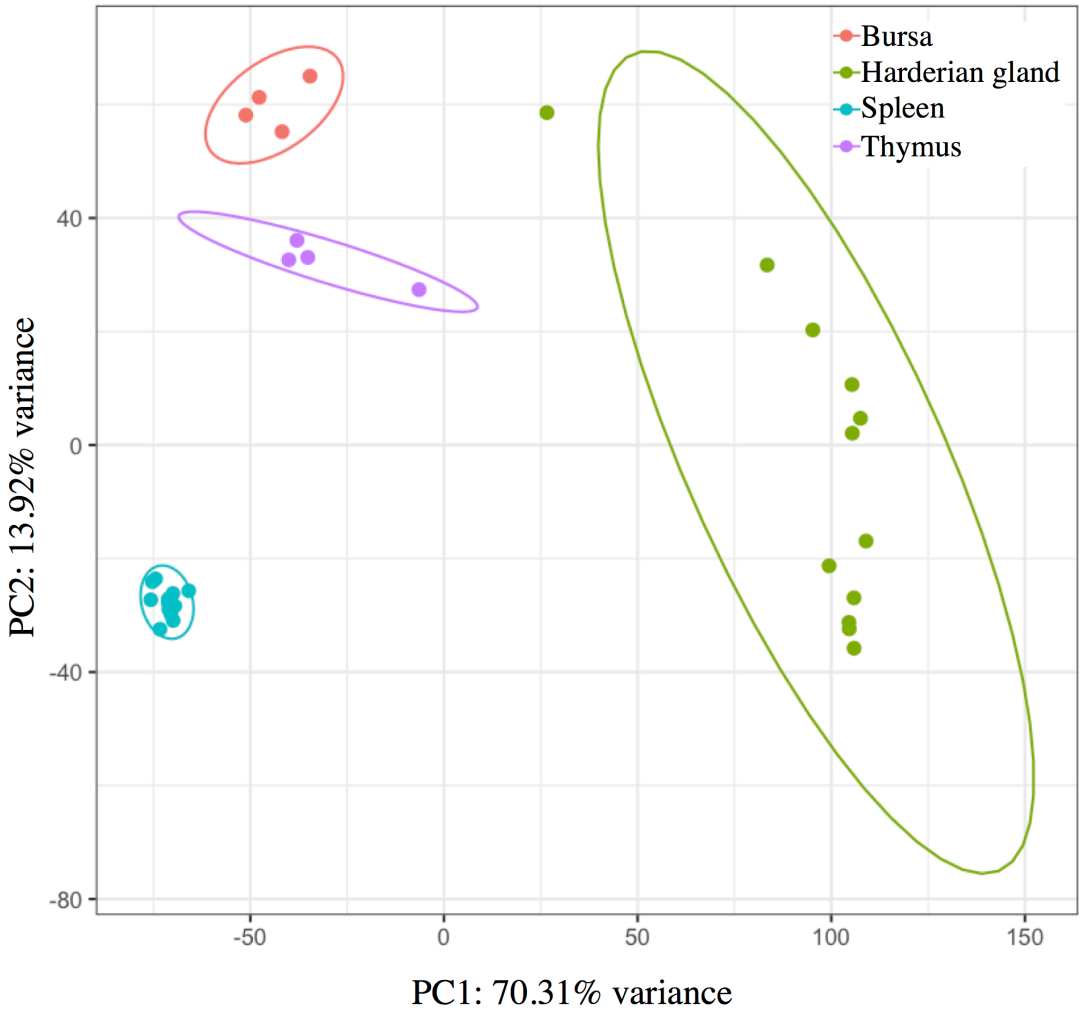

Figure 6-1: Principal component analysis shows clear clustering by tissue type. Each dot represents a tissue from an individual chicken. Principal component 1 (PC1) separated the Harderian gland samples (green) from the other immune tissues. Principal component 2 (PC2) separated the bursa (pink), thymus (purple), and spleen (blue). Ellipses were drawn with a 95% confidence. Plot was generated using pcaExplorer.

Relative expression analysis

The relative expression values followed a tri-modal distribution (mean = 1.676) (standard deviation = 3.997) (Figure 6-2). Gaps in the distribution may be related to the distinct clustering of the Harderian gland and other immune tissues (PC1=70.3%; Figure 6-1). A total of 143 transcripts were highly expressed in the non-Harderian immune tissues, whereas 2,386 transcripts were highly expressed in the Harderian gland. The 2,386 transcripts were input into Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA)

and of the 936 identified by IPA, 96 were classified as transcription regulators and 4 as translation regulators. Of the 143 transcripts highly expressed in the other immune tissues only 53 had an associated gene name. No significant GO terms were identified. Some immune genes of interest in the 143 transcripts included *C1QL3*, *C8A*, and *TLX1*. The relative expression analysis was more stringent than a differential expression analysis in which 99% of the transcripts were differentially expressed (false discovery rate < 0.05; data not shown).

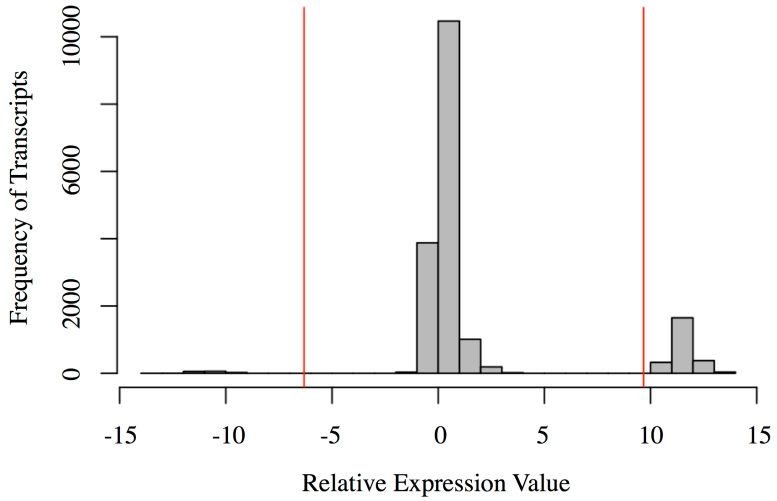

Figure 6-2: Histogram of the relative expression values for each transcript. Each bar represents the number of transcripts with a relative expression value within that range (x-axis). The red vertical lines represent two standard deviations below the mean (left) and above the mean (right). Transcripts to the left of the left red line are highly expressed in the other immune organs (spleen, bursa, thymus). Transcripts to the right of the right red line are highly expressed in the Harderian gland. Figure generated in R.

Gene set enrichment analyses

GO term analysis, pathway analysis, and network analysis were applied to the genes highly expressed in the Harderian gland on the assumption that these genes are the main drivers of functions that differentiate the Harderian gland from the other immune tissues. A cell type enrichment analysis (Shoemaker et al., 2012) showed no significant enrichment of any cell type based on these genes (data not shown); therefore, differences in expression levels among these tissues were likely not due to large differences in cell-type composition.

The top-layer GO terms identified by Panther for the genes highly expressed in the Harderian gland are shown (Figure 6-3). Most GO terms were related to development and morphogenesis. The most significant GO term was G-protein coupled receptor signaling (Figure 6-3). No classic immune related GO terms were identified, however, cell fate commitment, G-protein coupled receptor signaling, and cell-cell signaling may be immune related.

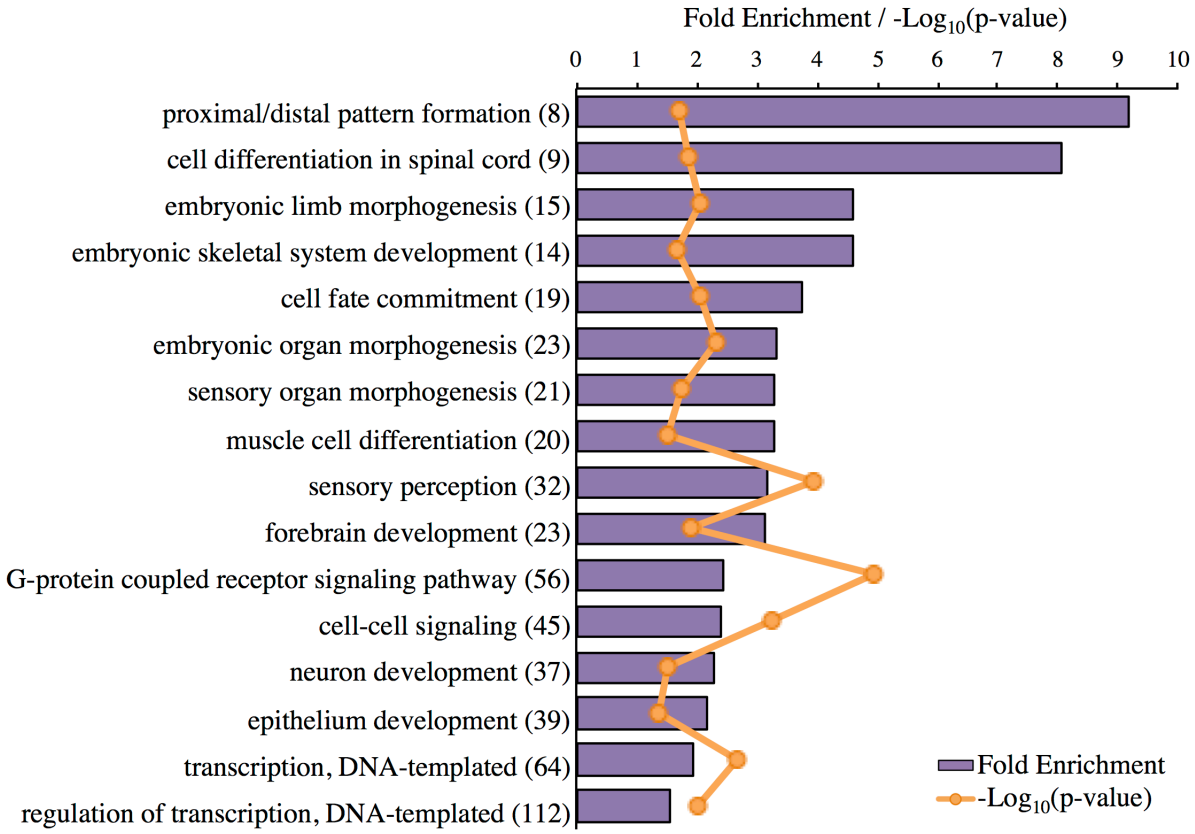

Figure 6-3: Significant GO terms associated with genes highly expressed in the Harderian gland. Transcripts highly expressed in the Harderian gland were converted to their corresponding gene ID using Ensembl (766 total) and input into Panther for statistical overrepresentation analysis using GO biological process (757 genes recognized and used for the analysis). The fold enrichment (purple bar) was calculated as the number of observed input genes divided by the number of expected genes based on the number of genes in the chicken genome. The number of genes associated with each GO term is listed to the right of the GO term in parenthesis. The Bonferonni correction for multiple testing was used to adjust p-values. The $-Log_{10}$(p-value) for each GO term is represented by orange markers.

IPA identified pathways associated with genes highly expressed in the Harderian gland (Table 6-2). IPA identified more immune related pathways than the Panther GO term

analysis (Figure 6-3). Notably, G-protein coupled receptor signaling was represented in both analyses (Figure 6-3, Table 6-2). Calcium Signaling, ILK signaling, CXCR4 signaling, Crosstalk between Dendritic Cells and Natural Killer Cells, and MIF Regulation of Innate Immunity are all pathways that have a direct relationship with the immune response.

Table 6-2: Canonical pathways associated with genes highly expressed in the Harderian gland

Ingenuity Canonical Pathways	p-value	z-score	genes
Calcium Signaling	0.0001	2.646	20
ILK Signaling	0.0005	3.771	19
Thrombin Signaling	0.0008	3	19
Actin Cytoskeleton Signaling	0.0028	3.873	19
Protein Ubiquitination Pathway	0.0141	---	19
Signaling by Rho Family GTPases	0.0072	4	18
G-Protein Coupled Receptor Signaling	0.0347	---	18
Tight Junction Signaling	0.0006	---	17
cAMP-mediated signaling	0.0112	4	17
Cardiac Hypertrophy Signaling	0.0186	3.207	17
Phospholipase C Signaling	0.0407	3.051	16
Cellular Effects of Sildenafil (Viagra)	0.0003	---	15
Epithelial Adherens Junction Signaling	0.0010	---	15
RhoGDI Signaling	0.0054	-3.464	15
Agranulocyte Adhesion and Diapedesis	0.0129	---	15
Mitochondrial Dysfunction	0.0115	---	14
Sertoli Cell-Sertoli Cell Junction Signaling	0.0158	---	14
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.0195	---	14
CREB Signaling in Neurons	0.0214	3.162	14
CXCR4 Signaling	0.0195	1.897	13
Aldosterone Signaling in Epithelial Cells	0.0219	---	13
GABA Receptor Signaling	0.0001	---	11
Gαi Signaling	0.0107	1.897	11
Ovarian Cancer Signaling	0.0363	---	11
Synaptic Long Term Depression	0.0407	3.317	11
Corticotropin Releasing Hormone Signaling	0.0166	3	10
RhoA Signaling	0.0324	3.162	9
Oxidative Phosphorylation	0.0363	---	9
Transcriptional Regulatory Network in Embryonic Stem Cells	0.0002	---	8
GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified...	0.0102	---	8
Crosstalk between Dendritic Cells and Natural Killer Cells	0.0302	---	8
Regulation of Actin-based Motility by Rho	0.0324	2.828	8
Glutamate Receptor Signaling	0.0087	---	7
Agrin Interactions at Neuromuscular Junction	0.0234	2.449	7
Remodeling of Epithelial Adherens Junctions	0.0234	---	7
Caveolar-mediated Endocytosis Signaling	0.0269	---	7
Basal Cell Carcinoma Signaling	0.0288	1.633	7
Serotonin Receptor Signaling	0.0079	---	6
Ethanol Degradation II	0.0174	---	5
Noradrenaline and Adrenaline Degradation	0.0234	---	5
MIF Regulation of Innate Immunity	0.0309	2.236	5
Triacylglycerol Biosynthesis	0.0398	---	5

The genes highly expressed in the Harderian gland formed a network with significantly more interactions than expected (p=6.6e-16). STRING identified two significant (FDR<0.05) KEGG pathways associated with these genes including Neuroactive ligand-receptor interaction (04080) and Tight junction (04530). Specific clusters of interest from the large network included Wnt genes (Figure 6-4A), GABA genes (Figure 6-4B), Heat shock proteins (Figure 6-4C), and G-protein coupled receptors (GPCR) (Figure 6-4D).

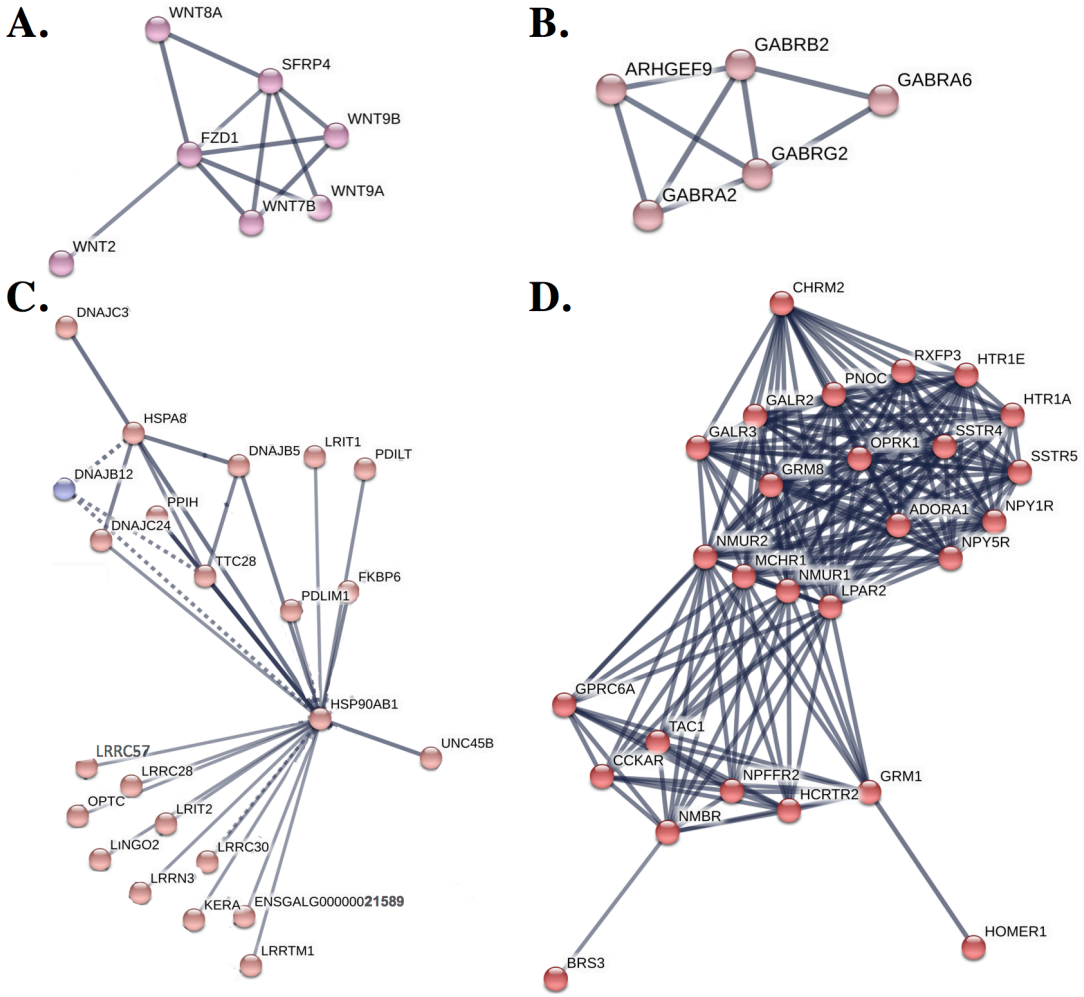

Figure 6-4: Network of genes highly expressed in the Harderian gland. Network generated by STRING. Edge thickness represents the confidence or strength of data support. A high confidence (0.700) cut-off was used to generate the network and all unconnected nodes were removed. Node color was based on MCL clustering (inflation parameter = 3). The high number of nodes and edges led to a large network. From that network four clusters of interest (A-D) were chosen to display.

A total of 48 miRNAs were highly expressed in the Harderian gland (Table 6-3), representing 39% of the miRNAs identified in this analysis. As the library construction kit used in this study employs a poly-A-tail selection, the reads that mapped to miRNA were likely from pre-processed miRNA. The correlation between the abundance of precursor miRNA and mature miRNA is dependent on the tissue and miRNA (Lee et al., 2008). It is possible that trace amounts of mature miRNA remained after poly-A-tail selection, but because the same kit was used for all tissues analyzed in this study, we assume no bias amongst tissues. It is also possible that reads belonging to the miRNA target sequence in regulated genes were incorrectly mapped to the miRNA itself. However, since reads that mapped to multiple places in the genome were removed, this is less likely.

Table 6-3: miRNAs highly expressed in the Harderian gland

miRNA	**Accession**[a]	**miRNA**	**Accession**[a]
gga-mir-1b	MI0001254	*gga-mir-451*	MI0004995
gga-let-7f	MI0001233	*gga-mir-454*	MI0006984
gga-let-7j	MI0001262	*gga-mir-1592*	MI0007319
gga-let-7a-1	MI0001234	*gga-mir-1640*	MI0007372
gga-mir-29b-2	MI0001266	*gga-mir-1684b*	MI0022501
gga-mir-29c	MI0001265	*gga-mir-1737*	MI0007476
gga-mir-30c-2	MI0001205	*gga-mir-1764*	MI0007506
gga-mir-30d	MI0001198	*gga-mir-1772*	MI0007515
gga-mir-34c	MI0001261	*gga-mir-1773*	MI0007516
gga-mir-101-2	MI0007558	*gga-mir-1800*	MI0007544
gga-mir-130a	MI0001241	*gga-mir-1812*	MI0007557
gga-mir-130b	MI0001239	*gga-mir-2126*	MI0010731
gga-mir-133a-2	MI0001248	*gga-mir-6546*	MI0022362
gga-mir-133b	MI0001206	*gga-mir-6580*	MI0022399
gga-mir-133c	MI0001255	*gga-mir-6606*	MI0022425
gga-mir-138-2	MI0001228	*gga-mir-6609*	MI0022428
gga-mir-144	MI0004996	*gga-mir-6614*	MI0022433
gga-mir-193b	MI0003698	*gga-mir-6653*	MI0022473
gga-mir-200b	MI0001250	*gga-mir-6663*	MI0022483
gga-mir-214	MI0008208	*gga-mir-6668*	MI0022488
gga-mir-221	MI0001178	*gga-mir-6677*	MI0022497
gga-mir-301a	MI0001240	*gga-mir-6701*	MI0022523
gga-mir-365b	MI0022403	*gga-mir-6704*	MI0022526
gga-mir-365-2	MI0003704	*gga-mir-7474*	MI0024147

[a]source mIRBase

Discussion

The immune related genes that were highly expressed in the Harderian gland are of particular interest because they show how the homeostatic state is different compared to the

bursa, spleen, and thymus. GPCR related genes, functions, and pathways were represented in every functional analysis. Every GPCR is activated by a specific ligand, which results in downstream signaling events. Glutamate is the ligand for several GPCRs identified as highly expressed in the Harderian gland, and IPA identified Glutamate Receptor Signaling as an impacted pathway. Glutamate is an amino acid that functions as a neuro-immuno-transmitter (Ganor and Levite, 2012). Glutamate can impact the immune system in several ways, i.e. affecting T cell survival, calcium levels, and cytokine expression levels (Ganor and Levite, 2012). Also, T cells, B cells, macrophages, and dendritic cells, express high levels of glutamate receptors (Ganor and Levite, 2012). The neurotransmitter GABA also acts as an immunomodulator (Jin et al., 2013). The genes highly expressed in the Harderian gland significantly impacted the GABA Receptor Signaling pathway and GABA receptors were represented in the network generated by STRING. GABA signaling can impact chemotaxis, phagocytosis, and cytokine secretion in immune cells (Jin et al., 2013). It is known there is a strong link between the central nervous system and the immune system (Black, 1994). The utilization of these neurotransmitters and their receptors in the Harderian gland compared to other immune tissues may be due to the focal role of local immunity in the Harderian gland, whereas, the other immune tissues in the current study are generally more important to systemic immunity.

Another GPCR, FZD1, is the receptor for Wnt proteins. Several Wnt genes were identified as highly expressed in the Harderian gland and were represented in the network analysis. The Wnt pathway is tightly regulated, as it is involved in development, cell differentiation, and the immune response (Staal et al., 2008). The miRNA mir-301a, has been shown to be activated by the Wnt pathway in glioma cells (Yue et al., 2016). Let-7 is also

involved in cell proliferation and differentiation (Roush and Slack, 2008). Previously, increased levels of let-7 pri-miRNA were found in undifferentiated embryonic stem cells (Wulczyn et al., 2007). Increased expression of Wnt genes and let-7 miRNAs in the Harderian gland may increase the efficiency of this lymphoid tissue, by serving as a home to progenitor or naïve lymphocytes that can quickly be differentiated or activated in response to a stimulus.

Small, non-coding, miRNAs regulate gene expression levels post-transcription. Many of the miRNAs identified as highly expressed in the Harderian gland influence immune related genes and pathways. In human cell lines, *mir-200b* was shown to inhibit the TLR4 pathway (Wendlandt et al., 2012), *mir-221* increased the expression of *NF-κβ* and *STAT3* (Liu et al., 2014), and *mir-193b* overexpression resulted in increased autophagy (Nyhan et al., 2016). In chickens, *mir-1764* decreased the expression levels of the inflammatory cytokine *STAT1* (Jeong et al., 2013), and *mir-30d* may regulate *IRF4* (Li et al., 2017). Avian influenza impacted the expression levels of several miRNAs in the chicken trachea and lung including the following miRNAs significant in the current study: *let-7a-1*, *let-7f*, *let-7j*, *mir-1b*, *mir-30d*, *mir-34c*, *mir-101-2*, *mir-144*, *mir-200b*, and *mir-451* (Wang et al., 2009). Increased expression of these miRNAs in the Harderian gland would clearly impact the immune response in this tissue. Using miRNAs for regulation purposes in a tissue that is required to respond quickly to pathogens that enter via the eye is a useful strategy. Since the Harderian gland transcriptome has not been analyzed until recently (Lamont, personal communication) and was not used to generate the reference genome, there were likely several Harderian gland specific lncRNAs and miRNAs not identified in this analysis. Further research is necessary to confirm the correlation of abundance between the likely precursor

miRNAs identified in this study and the mature miRNA levels, and to identify tissue specific, novel RNAs.

Overall this study identified 2,386 transcripts that were highly expressed in the Harderian gland compared to the bursa, thymus, and spleen of nonchallenged chickens. These transcripts highlighted the interaction between the central nervous system and the immune system via the neuro-immuno-transmitters glutamate and GABA. The Harderian gland may utilize *Wnt* genes and *let-7* miRNAs to control or stall cell differentiation. Previous studies have shown, several miRNAs identified as highly expressed in the Harderian gland have immune function. Moreover, these results elucidated the unique immune properties of the Harderian gland, a local immune tissue that must quickly respond to pathogens and vaccines that enter via the eye. It is important to gain a better understanding of the physiology of important avian tissues to develop better vaccines, breed for disease resistance in chickens, and potentially prevent zoonotic outbreaks.

Author contributions

MSD: collected data, processed RNA-seq data, executed analysis, data interpretation, wrote manuscript
SJL: oversaw data collection, analysis, and interpretation, read and reviewed manuscript

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